Universidade de Aveiro Departamento de Biologia
2014

Nuno Gonçalo de Carvalho Ferreira

O EFEITO DE QUÍMICOS EM ISÓPODES: UMA AVALIAÇÃO MULTI-ORGANIZACIONAL

THE EFFECTS OF CHEMICALS IN ISOPODS: A MULTI-ORGANIZATIONAL EVALUATION

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Loureiro, Investigadora Auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro e co-orientação do Professor Doutor Amadeu Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através de uma Bolsa de Doutoramento atribuída a Nuno Gonçalo de Carvalho Ferreira (BD/65739/2009)

Este trabalho é dedicado a toda a minha família mais próxima, a minha mãe Maria de Lurdes de Carvalho Ferreira, o meu irmão Ricardo Jorge de Carvalho Ferreira, à minha avó Emília Cabral de Carvalho e à memória do meu avô Álvaro Marques Ferreira, sem os quais não chegaria nem perto da pessoa que sou agora.

o júri

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Doutora Susana Patrícia Mendes Loureiro
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agradecimentos

“My dear, here we must run as fast as we can, just to stay in place. And if you wish to go anywhere you must run twice as fast as that.”

Lewis Carroll, Alice in Wonderland

Penso que não existe melhor descrição que a de Lewis Carroll para definir aquilo que para mim foi o meu doutoramento, não só pelo meu esforço, mas também pelos sentimentos que invoca de todos aqueles que me ajudaram e me apoiaram.

Ninguém nasce ensinado e ninguém por muito esforço que faça consegue sozinho aprender e avançar, por isso gostaria de agradecer aos meus orientadores por toda a ajuda. Ao Prof. Amadeu Soares pelo apoio que deu e pelos problemas que resolveu e à Doutora Susana Loureiro pela sua orientação, paciência e ajuda, mas também por aqueles “safanões” e “ puxões de orelhas” que foram necessários. Principalmente gostaria de agradecer por ter feito sempre mais do que tinha que fazer e por pensar mais além e me dar várias oportunidades para crescer cientificamente.

If I am grateful to all the work and support from my supervisors, the ones that also participated in it even for small amounts of time also deserve my gratefulness. Not only for their guidance but also for making me feel in Cardiff as in my second home. I could never thank enough Prof. Peter Kille and Prof. John Morgan for the welcome and that still remains in my memory as both come out from the main building door, with a smile as if they were welcoming a long long lost friend. I will always be grateful by their teachings in molecular Biology and in all other areas of Biology, but mainly by their never-ending encouragement to work and research. But Cardiff is not only two persons is also Luis, Raquel, Ceri, Dan, Yasser, Pierfrancesco, Mario, Pablo, Marta and many other people each one in their own way helping, integrating and welcoming me. I cannot forget also Jorge, the one that helped me out when I was there and that now is working with me side by side in Aveiro. And finally a special thanks to baby Mia, the one that can make you forget all the hard day work and just get hypnotized with her smile.

Como ninguém consegue fazer nada sem a ajuda do próximo, e eu não sou uma excepção agradeço a todos os co-autores deste trabalho por toda a ajuda que deram. Em especial queria agradecer ao Miguel Santos, uma pessoa excepcional que me ajudou no inicio quando tudo ainda era tudo um pouco novo para mim, pela suas capacidades extraordinárias de análise e de memória que conseguiam sempre ajudar onde falhava alguma coisa. Ao Diogo Cardoso, aquele que para mim ainda tem muito para mostrar, que esteve sempre ao meu lado para ajudar demorasse o trabalho 2h ou 12h a terminar. E por fim o não menos importante Rui Morgado, que trabalhou bastante em conjunto comigo, esteve horas infindáveis a trabalhar ao meu lado e do qual também pertence grande parte deste trabalho.

agradecimentos

Gostava de agradecer também àquela pessoa que esteve sempre no laboratório para ajudar no que fosse preciso o Abel Ferreira.

Também queria agradecer a uma longa lista de amigos que estiveram lá para me apoiar e para me ajudarem a descomprimir, ou a nos "grandes debates científicos", mesmo que me tenha afastado mais de alguns deles: Eva Oliveira, Andreia Rodrigues, Mário Seabra, Luis Korrodi, Rita Jordão, Eliana Cavaleiro, Sandra Gonçalves, Marco Fernandes, Hugo Monteiro, Cátia Santos, Patricia Silva, Rita Silva, Diana Campos, Fátima Simão, Tiago Batista, Fábio e Margarida Pratas, João Pestana, Sara Novais, Vanessa Oliveira, Luis Mendes, Maria Donas-Bôtto Bordalo, Hugo Vieira e Maria Pavlaki. E em especial queria agradecer à Sara Alves, principalmente por todo o apoio que deu neste último ano, e à Jeamylle Nilin àquela amiga que apesar de bastante longe e com um oceano enorme pelo meio conseguiu manter o contacto por skype e estar aqui deste lado sempre que necessário.

De todos aqueles que considero meus amigos tenho salientar em especial a Mónica Almeida, mais conhecida como a minha "esposa de trabalho", mas que no fundo foi e é mais que isso. É a pessoa que sempre lá esteve, que nunca deixou se de oferecer para o que quer que fosse necessário, nos bons e nos maus momentos, para nos rirmos e para discutimos a altos gritos e para no final de tudo deixarmos as nossas diferenças de lado e irmos relaxar um pouco. Aquela que "segurou muitas vezes tudo, enquanto eu reparava o pilar para o castelo não desabar".

Para o fim deixa-se sempre quem mais se quer pois sabemos não nos vamos esquecer delas, a minha família, em especial aqueles a quem dedico também esta tese à minha mãe, irmão e à minha avó, que sabem bem o quanto os ADORO e quanto me é difícil expressar por palavras. Obrigado por estarem sempre presentes em todos os momentos maus e bons, em todas as partidas e chegadas e pelo apoio sempre dado.

palavras-chave

Isópodes terrestres, biomarcadores, nickel, dimethoato, avaliação de risco ambiental, avaliação multi-organizasional

resumo

Os efeitos negativos de contaminantes de origem antropogénica que aparecem no meio ambiente têm um impacto negativo muitas vezes em organismos considerados benéficos. Dentro deste grupo de organismos podemos incluir os isópodes terrestres, detritivorous, cuja função se baseia na fragmentação de matéria vegetal, que poderão posteriormente ser facilmente colonizados por bactérias e os seus nutrientes incorporados nos solos.

Para avaliar os efeitos de contaminantes em isópodes terrestres, mais concretamente na espécie *Porcellionides pruinosus*, foram usados como modelos o metal níquel e o pesticida dimetoato, fazendo uma abordagem em diferentes níveis organizacionais. O trabalho iniciou-se pela avaliação do impacto a nível das vias de destoxificação (biomarcadores enzimáticos) e reservas energéticas (quantificação das reservas, consumo energético e alocação da energia celular), juntamente com alterações a nível dos indivíduos e da população (mortalidade), passando por uma análise de metabolómica (usando 1H-RMN de líquidos) e finalmente uma análise da expressão génica (transcriptoma e RT-qPCR). De forma a melhor entender as variações que podem ocorrer oriundas de variações de fatores abióticos, foi também realizado um estudo sob os efeitos da variação de temperatura, humidade do solo e radiação UV em isópodes terrestres.

A avaliação feita a nível dos biomarcadores bioquímicos, quantificação das reservas e parâmetros energéticos demonstrou que o aumento da temperatura iria afectar negativamente os organismos devido ao stress oxidativo gerado. Tendo em conta que esta espécie está aclimatada a ambientes com solos com humidades baixas, a exposição a solos com humidades superiores mostrou a existência de um pequeno intervalo entre o que são consideradas condições óptimas e condições bastante adversas, sendo aí verifica uma elevada mortalidade. Os efeitos da radiação UV demonstraram que as doses que actualmente chegam à superfície do planeta constituem uma ameaça para os isópodes terrestres.

A avaliação feita à toxicidade do dimetoato em biomarcadores bioquímicos, reservas e parâmetros energéticos, demonstrou que além da toxicidade já esperada (inibição da enzima acetilcolinesterase), este stressor induz igualmente stress oxidativo. Este efeito foi observado para ambas as concentrações usadas (dose recomendada de aplicação em campo e dose próxima do EC50) e que a sua combinação com diferentes temperaturas (20ºC

resumo

e 25ºC) dava origem a diferentes padrões de resposta. Também foi observado que a taxa de degradação do dimetoato era superior em solos com a presença de isópodes.

Num estudo semelhante ao anterior, realizado com níquel, foi observada toxicidade induzida por stress oxidativo. No entanto os organismos expostos a este stressor apresentam uma estratégia em que os custos energéticos associados à destoxificação (biomarcadores bioquímicos) parecem ser compensados com alterações positivas a nível dos parâmetros energéticos.

Neste trabalho é apresentado pela primeira vez o perfil metabolómico de isópodes terrestres expostos a dimetoato e níquel, sendo que até à data apenas um estudo tinha apresentado o perfil metabólico de isópodes em situação de homeostasia. Na primeira parte do estudo é apresentado o perfil metabólico que identifica 24 novos metabolitos que ainda não tinham sido descritos anteriormente. Numa segunda parte é apresentada uma variação do perfil de organismos não expostos ao longo do ensaio e finalmente são apresentadas as variações metabólicas em organismos expostos a dimetoato e a níquel. Os resultados mostraram que ambos os stressores causaram alterações que eram dependentes da concentração e do tempo. Quando expostos a níquel os organismos evidenciam alterações ao nível do crescimento, muda e síntese de hemocianina e glutationas, alterações ao nível das vias energéticas e na osmorregulação. Em relação aos efeitos da exposição a dimetoato, foram observadas alterações a nível da osmorregulação, das vias energéticas e na muda, mas também ao nível da neurotransmissão.

Neste trabalho é apresentado pela primeira vez um transcriptoma completo de um isópode terrestre, da espécie *Porcellionides pruinosus*. Este transcriptoma veio complementar a atual, mas parca, informação disponível sobre este grupo de organismos. Tendo como base o transcriptoma, foi posteriormente realizada uma análise de RNA-Seq e de RT-qPCR. A análise de RNA-Seq foi realizada apenas em organismos expostos a níquel e mostrou que este stressor tem um impacto ao nível genético e epigenético, no transporte, acumulação e eliminação de metais, gera stress oxidativo, neurotoxicidade e afecta também a reprodução. Estes resultados foram confirmados pela análise feita através de RT-qPCR. Em relação aos efeitos de dimetoato nestes organismos a nível genómico, a sua avaliação foi apenas realizada através de RT-qPCR. Foi observado que este stressor gera stress oxidativo, neurotoxicidade, tem um impacto em marcadores epigenéticos, na reparação de ADN e provoca alterações a nível da divisão celular.

Este estudo permitiu a realização de uma “Adverse Outcome Pathway” (AOP) que poderá ser usada mais tarde para fins legislativos.

keywords

Terrestrial isopods, biomarkers, nickel, dimethoate, environmental risk assessment, multi-organizasional level evaluation

abstract

The global aim of this thesis was to evaluate and assess the effects of a pesticide (dimethoate) and a metal (nickel), as model chemicals, within different organization levels, starting at the detoxification pathways (enzymatic biomarkers) and energy costs associated (energy content quantification, energy consumption and CEA) along with the physiological alterations at the individual and population level (mortality), leading to a metabolomic analysis (using liquid 1H-NMR) and finally a gene expression analysis (transcriptome and RT-qPCR analysis). To better understand potential variations in response to stressors, abiotic factors were also assessed in terrestrial isopods such as temperature, soil moisture and UV radiation.

The evaluation performed using biochemical biomarkers and energy related parameters showed that increases in temperature might negatively affect the organisms by generating oxidative stress. It also showed that this species is acclimated to environments with low soil moisture, and that in high moisture scenarios there was a short gap between the optimal and adverse conditions that led to increased mortality. As for UV-R, doses nowadays present have shown to induce significant negative impact on these organisms.

The long-term exposure to dimethoate showed that besides the neurotoxicity resulting from acetylcholinesterase inhibition, this stressor also caused oxidative stress. This effect was observed for both concentrations used (recommended field dose application and a below EC50 value) and that its combination with different temperatures (20ºC and 25ºC) showed different response patterns. It was also observed that dimethoate's degradation rate in soils was higher in the presence of isopods.

In a similar study performed with nickel, oxidative stress was also observed. But, in the case of this stressor exposure, organisms showed a strategy where the energetic costs necessary for detoxification (biomarkers) seemed to be compensated by positive alterations in the energy related parameters.

In this work we presented for the first time a metabolomic profile of terrestrial isopods exposed to stressors (dimethoate and niquel), since until the moment only a previous study was performed on a metabolomic evaluation in non-exposed isopods. In the first part of the study we identify 24 new metabolites that had not been described previously. On the second part of the study a metabolomic profile variation of

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non-exposed organism throughout the exposure was presented and finally the metabolomic profile of organisms exposed to dimethoate and nickel. The exposure to nickel suggested alteration in growth, moult, haemocyanin and glutathione synthesis, energy pathways and in osmoregulation. As for the exposure to dimethoate alterations in osmoregulation, energy pathways, moult and neurotransmission were also suggested.

In this work it was also presented the first full body transcriptome of a terrestrial isopod from the species *Porcellionides pruinosus*, which will complement the scarce information available for this group of organisms. This transcriptome also served as base for a RNA-Seq and a RT-qPCR analysis. The results of the RNA-Seq analysis performed in organisms exposed to nickel showed that this stressor negatively impacted at the genetic and epigenetic levels, in the trafficking, storage and elimination of metals, generates oxidative stress, inducing neurotoxicity and also affecting reproduction. These results were confirmed through RT-qPCR. As for the impact of dimethoate on these organisms it was only accessed through RT-qPCR and showed oxidative stress, an impact in neurotransmission, in epigenetic markers, DNA repair and cell cycle impairment.

This study allowed the design of an Adverse Outcome Pathway draft that can be used further on for legislative purposes.

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CHAPTER I

General Introduction and Objectives

1. Introduction

The full extension of soil importance goes beyond its use for anthropogenic activities (growing food, planting trees for oxygen and wood or for infrastructures), as soil provides all the conditions for plants such as water, nutrients or even anchorage; provides habitat for a large number of organisms such as annelids, isopods, fungi or collembolans, among many others, that play an essential role in carbon and nutrient cycling or even bacteria for the carbon sequestration. Soil also acts as a buffer in the regulation of water between the ground and the atmosphere, or buffer for temperature changes and finally due to soil's exchange properties, it also acts as pH buffer, nutrients and other elements loss by leaching or volatilization (Wild, 1993).

As stated in "The European environment – state and outlook 2010" published by the European Environment Agency regarding soil, its use and the importance within the European Union (EU):

"Nearly all of the food and fibres used by humans are produced on soil. Soil is also essential for water and ecosystem health. It is second only to the oceans as a global carbon sink, with an important role in the potential slowing of climate change. Soil functions depend on a multitude of soil organisms which makes it an important part of our biodiversity. Nevertheless, soil in many parts of Europe is being over-exploited, degraded and irreversibly lost due to impacts from industrial activities and land use change, leading to soil sealing, contamination, erosion and loss of organic carbon. Due to these problems, legislation for the protection of soils has been proposed at EU level."

(EEA, 2010)

This small but well defined introductory idea, shows not only the importance of soil, but also brings out the problems that should be faced in order to maintain this important resource. In the report, soil degradation is resulting from water and wind erosion (approx. 16% of Europe's total land), decline in organic matter (about 45% of soils have low or very low organic matter), high soil compaction (32% to 36% of soils being highly vulnerable), high sealing due to urban sprawl and transport infrastructures (an increase of 6% was observed

between 1990-2000), salinization resulting from agricultural practices of irrigation and appliance of fertilizers (3.8 million ha), contamination (more than 3 million sites are estimated to be potentially polluted) and biodiversity decline as a result of all the above mentioned processes (EEA, 2010).

Environmental Risk Assessment (ERA) is one of the most important, debated and focused points of investigation. When talking about ERA, we are talking not only about preserving the environment from the adverse effects induced by man, as the increase of contamination by releasing into the environment xenobiotics from urban communities, agriculture or industry, but also how to prevent that some of that contamination returns to us through food.

To deal and prevent some of these negative points, as for example contamination or biodiversity loss, the European legislation REACH (Registration, Evaluation, Authorization and restriction of Chemicals) was implemented in the 1st June 2007, and aims at providing protection to human health and the environment from the use of chemicals, by regulating the use of all compound produced or transformed within the European countries (Commission, 2006). Another important legislation implemented is the Commission regulation (EU) No 283/2013 that will be implemented by the 1st January 2014 for active substances, and the 1st January 2016 for plant protection products (EU, 2013). This legislation aims at setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. The use of fast methods for the evaluation of toxicity would greatly be appreciated and would improve the delivery of the results from this evaluation. The majority of the evaluation tests used is based on phenotypical observations (e.g. mortality or reproduction) that provide information on the organism status, and sometimes, can be transposed to the population. These assays although ecologically more relevant and sensitive at lower concentrations are more time consuming and therefore more expensive, which supports again the necessity of using faster evaluation methods. But to develop these new methods is necessary to evaluate and have a more accurate understanding of the mode of action (MoA) of stressors and which pathways they affect. In fact to be able to do short term exposures (less than 7 days) is advised to go first for long-term exposures, in order to evaluate not only low effect concentrations (LOEC), but also non-effect concentrations (NOEC), and determine the most relevant sampling time(s).

Therefore the use of biomarkers to evaluate more accurately effects, ranging from molecular biomarkers to physiological biomarkers (Fig. 1.1), would serve the desire proposes. Although a deep investigation is needed before the implementation of these biomarkers, this work may be a step forward for its implementation.

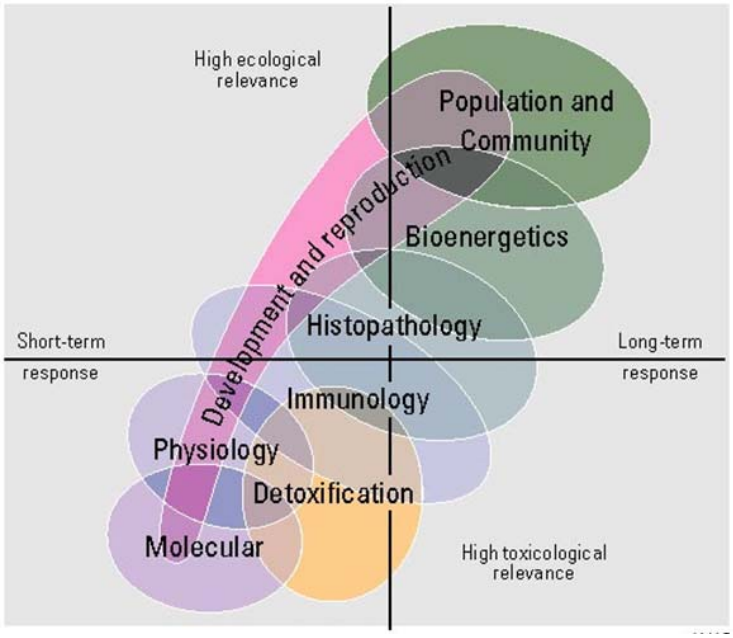

Fig. 1.1 Relationship between levels of biological organization, processes where effects of toxicants can be measured, time of response and ecological/toxicological relevance (from http://www.amap.no/).

The development of biomarkers for the previous stated evaluation cannot be performed in a general way and applied to all organisms and environments without any discrimination. In fact, it is necessary to select appropriated species that will have very specific and characteristic traits, which will be helpful in monitoring these environments. In the next section it is described the order Isopoda and their main ecological characteristics and features, which make them suitable to be used in ecotoxicological approaches and risk assessment.

2. Terrestrial isopods and exposure routes in Ecotoxicology

Terrestrial isopods are commonly known as woodlice, pill bugs, slaters or sowbugs and taxonomically are included in the order Isopoda. They are crustaceans that successfully occupied terrestrial habitats, and are more related to crabs or lobsters than to terrestrial arthropods such as insects, spiders or centipedes (Lokke and vanGestel, 1998).

Terrestrial isopods are found in almost all soil ecosystems, mainly in temperate habitats, and play a very important role in those ecosystems. They feed on decayed organic matter being part of the macrodecomposer community structure. Processes such as aeration, drainage and incorporation of the degraded organic matter mediated by this invertebrate group are also important key features that enhance soil quality, nutrient recycling, structure maintenance and fertility (Drobne, 1997; Paoletti and Hassall, 1999). Isopod species present nocturnal activity and their most important predators are beetles, spiders, centipedes, toads, shrews and birds (Lokke and vanGestel, 1998). As cryptozoic organisms, they are characterized for presenting an aggregating behavioural response, due to the production of an aggregation pheromone (Takeda, 1980), hiding during the day under stones, bark or even thick leaf litter in places with high humidity (Lokke and vanGestel, 1998).

The use of terrestrial isopods in ecotoxicology has already been adopted within the scientific community since early 80's, due to their capacity to handle high amounts of metals (Ribeiro et al., 1999), but also because they can detect the presence of contaminants (Loureiro et al., 2005). Regarding the exposure to metals, one of the reasons for their use is the capability to assimilate high amounts from the environment (Donker et al., 1990; Drobne, 1997; Hopkin, 1990) and their unique processes of accumulation and excretion that occurs in the hepatopancreas cells (Morgan et al., 1999; Tarnawska et al., 2007). Another important feature present in these organisms is the hard exoskeleton that limits the exposure route to chemicals only through dietary exposure (food or/and soil - Vijver et al., 2005) and via the pleopods structures that are responsible for the absorption of water from soil by capillary action (Sutton, 1980).

The species *Porcellionides pruinosus* has already been used in several studies as they are frequent in southern Europe, but also in tropical regions (like Brazil) and are active

throughout the year; also they are easily hand collected and are easy to maintain in laboratory cultures, where they are able to complete their life-cycle. This species has also proven to be a good indicator for the effects of pesticide and metal contamination in soils (Loureiro et al., 2009; Loureiro et al., 2005; Santos et al., 2010a).

They typically present a flat ventral-dorso, and a segmented body, with rigid exoskeleton and jointed limbs. Although in early stages of development the segments resemble each other, when adults a total of three groups can be identified: the head, followed by the thorax or pereion, and finally the abdomen or pleon (Fig. 1.2 - adapted from Sutton, 1980).

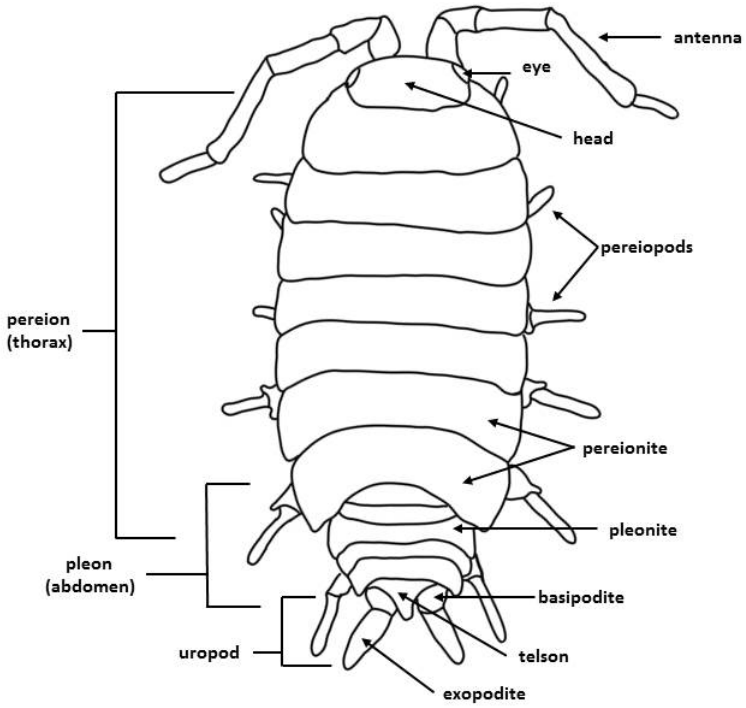

Fig. 1.2 Dorsal view of *Porcellionides pruinosus*, with the respective segmented structure. In the left side is described the three segmented parts of the body, and on the right side is described every specific structure (adapted from Sutton, 1980).

Briefly, the digestive system (Fig. 1.3) of these organisms is composed by a strait gut connected in the upper part by four branches of lobes (two for each side) called hepatopancreas (Sutton, 1980).

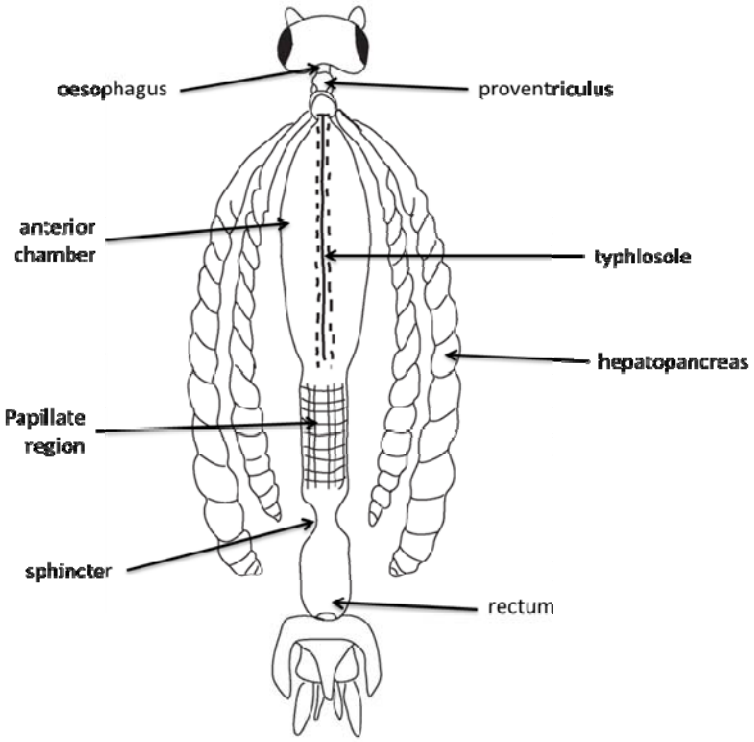

Fig. 1.3 Scheme for the dorsal view of the dissected digestive system (from Drobne and Kralj-Iglič, 2009)

The hepatopancreas is a 4-tube organ, comprised of two very different metal-sequestering cell types handling Cu and Fe. The '*S*' cell is a small and cone shaped cell, containing cuprosomes (Cu and S-rich organelles) that participate in haemocyanin synthesis (Köhler et al., 1996; Morgan et al., 1990) and with a long term residence time. The other cell type, the '*B*' cell, is large, with Fe (and PO_4-rich) inclusions, and it is characterized by discharging lipid and Fe contents diurnally in a feeding cycle (Hames and Hopkin, 1989; Tarnawska et al., 2007). The purpose of this '*B*' cell cycle is presently unknown. The '*B*' cells undergo a striking diurnal cycle of ultrastructural changes in which the contents of the apical cytoplasm are voided in an apocrine manner over ~11 hours. Subsequently, a recovery phase lasts the remainder of the 24 hour period, during which the cytoplasm is recharged with lipid,

glycogen and Fe rich inclusions (Hames and Hopkin, 1991). Thus, there is a continuous daily cycle of Fe acquisition and excretion from '*B*' cells (

Fig. **1.4**).

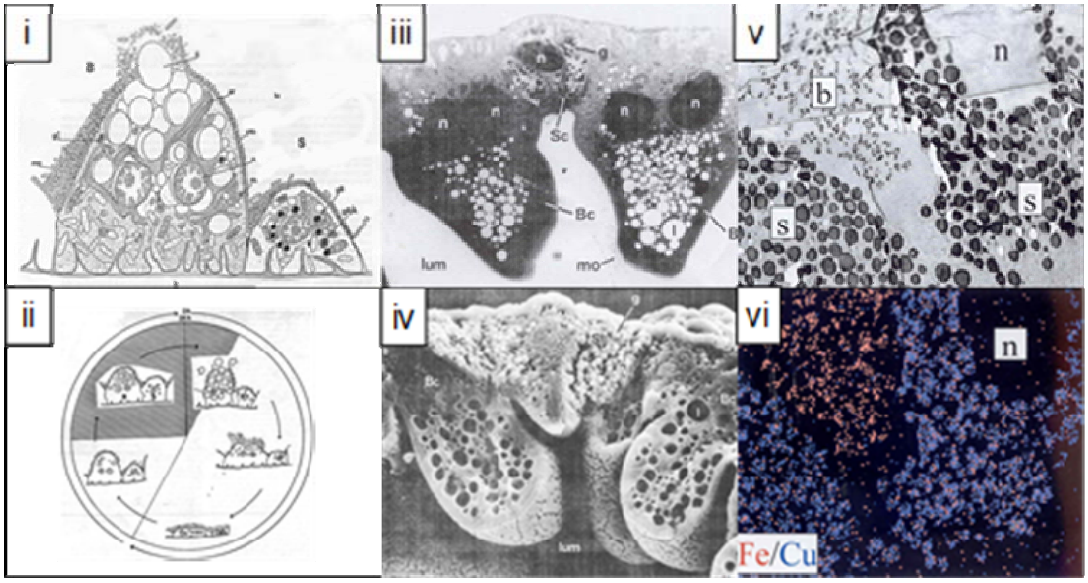

Fig. 1.4 (i) A sketch of the larger, binucleate '*B*' cell and the small '*S*' cell. The '*B*' cell has many lipid droplets in its apical cytoplasm while the '*S*' cell contains dense cuprosomes. The apical (lumenal) surface of both cells has a brush border, while their basal surfaces are separated from the haemocoel by infolded basement membranes (from Hames and Hopkin, 1991). (ii) The diurnal cycle of apocrine secretion of Fe and lipid from the '*B*' cell (from Hames and Hopkin, 1991). (iii) A light micrograph of '*S*' and '*B*' cells from the hepatopancreas (from Hopkin and Martin, 1982). (iv) A scanning electron micrograph of a freeze-fractured hepatopancreas emphasising the cuprosomes in the '*S*' cell (from Hopkin and Martin, 1982). (v) and (vi) A transmission electron micrograph and matching dispersive X-ray distribution maps for Cu (blue) and Fe (red) of two '*S*' cells (with Cu-laden cuprosomes) and a wedge of '*B*' cell cytoplasm (with Fe vesicles - from Morgan et al., 1990).

3. Biomarkers

The definition of biomarker is not consensual and varies not only from author to author but also in the scientific area where it is applied. The first concept was presented in the 70's in a medical context, and then around the 90's it became a main focus area for environmental studies (Depledge and Fossi, 1994; Walker, 1992).

A definition for the term "biomarker" in environmental studies can be considered as follows: "*Biomarker is any biological response to a stressor that can demonstrates any deviation from the normal organism life status*". Although a little more simplistic than the normal definitions, it includes all associated and predominant features.

This definition starts by referring "*any biological response*", which does not specify "at a sub-individual level" which was included in definitions such as the one from Mendelsohn et al. (1998), and therefore can include individual responses such as behaviour (Bayley et al., 1999). These biological responses will then include measurements at a molecular level (e.g. epigenetics, genomics, proteomics and metabolomics), subcellular, cellular and systemic levels (e.g. physiological or immunological alterations), behavioural level (e.g. avoidance, aggregation, velocity, movement) and even organism products (e.g. urine, faeces, hair, feathers, etc.). The definition also includes "*to a stressor*", which may differ from previous definitions where biomarkers are only connected with xenobiotics. But not only xenobiotic can induce changes in biomarkers, therefore resulting into alterations for ecosystems; abiotic factors, such as temperature, ultraviolet radiation (UV-R) or moisture among others can also affect ecosystems, in the same way and extent as anthropogenic contaminants. The non-inclusion of higher level endpoints in this definition, as for example growth or reproduction, is related to the two predominant features that are present in the definitions of all authors: they need to give an accurate relationship between the stressor(s) and the response and they have to be sensitive, and give quick responses, to serve as early alarms regarding the impact of the stressor(s) on organisms (Morgan et al., 1999).

3.1. Biomarkers at a genome level: Transcriptome and real-time PCR

With the rapid increase in technology and the decrease in its costs, nowadays it is possible to analyse gene expression in such a way that was not possible some years ago. This analysis can be made gene by gene using real-time PCR (RT-qPCR) or globally through RNA-Sequencing (RNA-Seq) after transcriptome sequencing.

The transcriptome is a complete set of transcripts (mRNAs, non-coding RNAs and small RNAs) in a cell that exists in a specific moment for a specific condition (Wang et al., 2009). The transcriptome is the template that will serve for the synthesis of proteins, providing therefore the most distinctive functions within the organism (Hegde et al., 2003).

To compare the transcriptome from organisms in different conditions an RNA-Seq analysis is performed. Briefly, this analysis consists on the conversion of RNA (from a single cell, a tissue or even the full organism) to a library of cDNA, and the attachment of some adaptors that will allow its high throughput sequencing by one end (single-end sequencing) or by both ends (pair-end sequencing). This sequencing will then result into fragments with different base-pairs (Table 1.1) and using different requirements as presented in the work of Quail et al. (2012). This fragments will then be assembled using different pipelines that already exist in the market (e.g. CLC Genomics®, Velvet (Zerbino and Birney, 2008), Abyss (Simpson et al., 2009), Trinity (Grabherr et al., 2011) and each fragment will be identified to each correspondent gene.

Another technique that can be used at a genomic level is the individual gene RT-qPCR analysis. This technique is based on quantifying differences in gene expression levels between samples (Taylor et al., 2010). By using a fluorescent dye that will intercalate with double-stranded DNA or by fluorescent labelled oligonucleotides hybridization, it is possible to amplify and simultaneously quantify the targeted DNA molecule (VanGuilder et al., 2008). Plotting fluorescence against the number of cycles on a logarithmic scale allows the determination of the number of cycles necessary for the different samples to obtain the same amount of amplified DNA (VanGuilder et al., 2008).

Table 1.1 Technical specifications of Next Generation Sequencing platforms presented in the work of Quail et al. (2012)

Platform	Illumina MiSeq	Ion Torrent PGM	PacBio RS	Illumina GAIIx	Illumina HiSeq 2000
Instrument Cost*	$128 K	$80 K**	$695 K	$256 K	$654 K
Sequence yield per run	1.5-2Gb	20-50 Mb on 314 chip, 100-200 Mb on 316 chip, 1Gb on 318 chip	100 Mb	30Gb	600Gb
Sequencing cost per Gb*	$502	$1000 (318 chip)	$2000	$148	$41
Run Time	27 hours***	2 hours	2 hours	10 days	11 days
Reported Accuracy	Mostly > Q30	Mostly Q20	<Q10	Mostly > Q30	Mostly > Q30
Observed Raw Error Rate	0.80 %	1.71 %	12.86 %	0.76 %	0.26 %
Read length	up to 150 bases	~200 bases	Average 1500 bases**** (C1 chemistry)	up to 150 bases	up to 150 bases
Paired reads	Yes	Yes	No	Yes	Yes
Insert size	up to 700 bases	up to 250 bases	up to 10 kb	up to 700 bases	up to 700 bases
Typical DNA requirements	50-1000 ng	100-1000 ng	~1 µg	50-1000 ng	50-1000 ng

* All cost calculations are based on list price quotations obtained from the manufacturer and assume expected sequence yield stated.
** System price including PGM, server, OneTouch and OneTouch ES.
*** Includes two hours of cluster generation.
**** Mean mapped read length includes adapter and reverse strand sequences. Subread lengths, i.e. the individual stretches of sequence originating from the sequenced fragment, are significantly shorter.

3.2. Biomarkers at a metabolomic level: Nuclear Magnetic Resonance (NMR)

The high-resolution nuclear magnetic resonance spectroscopy (NMR), in particular 1H NMR is being extensively used to establish metabolomic profiling from clinical samples to plants, vertebrates and invertebrates, microorganisms or fungi (e.g. Beckwith-Hall et al., 2002; Gibb et al., 1997; Lenz et al., 2005; Tuffnail et al., 2009; Ward et al., 2003). This technique can detect at the same time all proton bearing compounds in the sample, giving information about the presence in the samples of amino acids, lipids, carbohydrates, even organic and fatty acids, amines, esters or ethers, and even allow their quantification (Ward et al., 2003). Some advantages of using this technique include short analysis time to obtain a spectra result with a minimal sample preparation along with a non-destructive analysis that allows the use of the sample for other techniques, an excellent ability to quantify metabolites present in the sample and in most of the cases a good ability to identify them. This last point may also be one of the cons of this technique, as complex samples give rise to "crowded spectra" which will require the running of 2D NMR techniques or a higher statistical analysis of the samples. Other two important cons of these techniques is their sensitivity, where for example small changes in pH may lead to inadequate comparison of results between samples, and the concentrations required may be in some cases significantly high (Viant and Sommer, 2013).

3.3. Biomarkers at a biochemical and cellular level:

Acetylcholinesterase (AChE)

The enzyme acetylcholinesterase (AChE) is an enzyme responsible for the hydrolysis and inhibition of the neurotransmitter acetylcholine, into choline and an acetate group (Fig. 1.5), found on the anterior part of nerve terminals (Purves et al., 2008). The inhibition of this enzyme greatly affects the target organism as AChE plays multiples and unrelated biological functions (Soreq and Seidman, 2001). This inhibition can lead to symptoms such as multiple system atrophy or deterioration of cognitive autonomic and neuromuscular functions and in extreme even to respiratory failure (Soreq and Seidman, 2001).

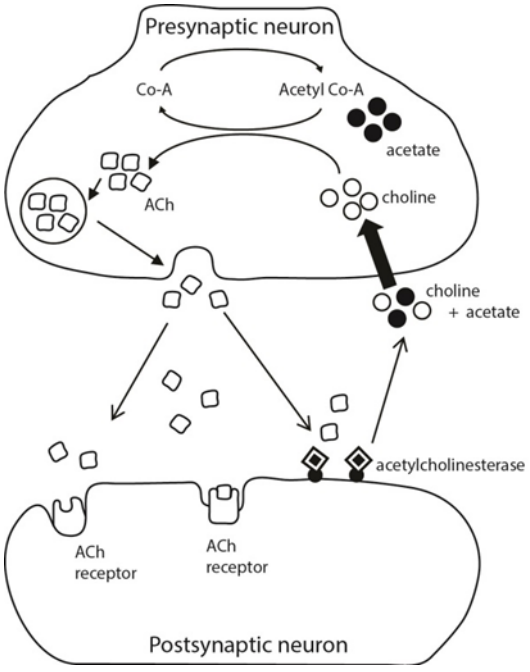

Fig. 1.5 The cholinergic synapse scheme. The synthesis of acetylcholine (**ACh**) through the conjugation of acetyl coenzyme A with choline occurs in the presynaptic neuron using. After the synthesis ACh is packaged into vesicles and released into the synaptic cleft. Here it binds to muscarinic receptors and then hydrolysed by acetylcholinesterase (**AChE**) into choline. This step leads to the transitory formation of acetyl-AChE which is going to suffer another hydrolysis releasing the acetate.

The use of this enzyme activity as biomarker has already been use for field and laboratory studies to assess exposure for example to organophosphates and carbamate insecticides (Drobne et al., 2008; Santos et al., 2011; Santos et al., 2010b; Stanek et al., 2006), or metals (Calisi et al., 2009; Elumalai et al., 2007).

The characterization and activity range measurements of AChE is essential for a good determination of its toxicity as cholinesterases can be divided into several classes and these classes can provide the same cleavage as the AChE (Bocquene et al. 1990, Garcia et al. 2000). In the case of the species *Porcellionides pruinosus* this characterization has already been made by Ferreira et al. (2010), where a description of the cholinesterase form present in this species is described has being acetylcholinesterase.

Lactate dehydrogenase (LDH)

Lactate dehydrogenase is an enzyme that plays an important role in several metabolic pathways (Fig. 1.6), being part of the glycolysis as the terminal enzyme that is responsible for the reduction of pyruvate to lactate and also important in the redox maintenance (Amiard-Triquet et al., 2012). The measurement of LDH activity as biomarker has already been used in several ecotoxicological studies (e.g. Coelho et al., 2011; Domingues et al., 2010; Monteiro et al., 2007), and regarding the case-studies with invertebrates an increase of its activity is normally observed upon stress (e.g. Diamantino et al., 2001; Ribeiro et al., 1999).

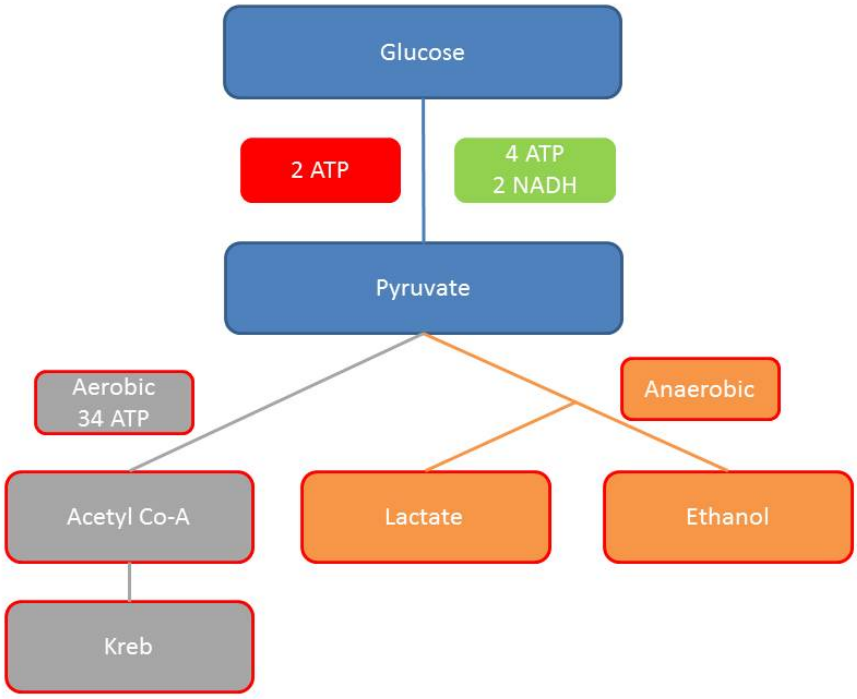

Fig. 1.6 Simplified scheme of glucose transformation into energy. The glycolysis consumes 2 ATP and gives origin to 4 ATP and 2 NADH, by transforming the glucose into pyruvate. This pyruvate can then follow the aerobic route giving origin to 34 ATP, or the anaerobic one not originating any ATP. The transformation from pyruvate into lactate is mediated by the enzyme lactate dehydrogenase, and the incorporation of pyruvate into the Krebs cycle is through the pyruvate dehydrogenase and acetyl Co-A.

Oxidative stress

The incomplete reduction of oxygen (e.g. superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$)) generates chemical species that are generally called reactive oxygen species (ROS). An organism that shows an imbalance of ROS and cannot handle this situation in a fast and effective way, is described as being under oxidative stress (D'Autreaux and Toledano, 2007). As ROS may be generated in the electron transport chain (ETC) and be important for its function, they may also result from other sites within or outside the cell and are handled by mechanistic detoxification pathways that include enzymes such as glutathiones or catalase (Cadenas and Davies, 2000). In Fig. 1.7 it is shown a simple scheme on how ROS are handled by distinct enzymes and how do they cause damage within the organism (Davies, 2000).

One of the first enzymes that enter the detoxification pathways are the glutathione *S*-transferases (GST) enzymes. The GST act as catalysts for the conjugation of various electrophilic compounds with the tripeptide glutathione (Armstrong, 1987; Gulick and Fahl, 1995). Their role is to serve has carrier proteins increasing the available lipophilic toxicants in phase I enzymes or by binding to electrophilic compounds reducing the likelihood of these compounds to bind to other macromolecules such as DNA (Schelin et al., 1983).

Another detoxification enzyme responsible for handling the ROS is catalase (CAT) which facilitates the removal of H_2O_2 from the organism. This enzyme is associated with the peroxisomes that function on the fatty acid metabolism (Huggett et al., 1992), and it also appears to be connected along with the glutathione peroxidase (GPx) activity to combat the oxidant stress exposure (Diesseroth and Dounce, 1970).

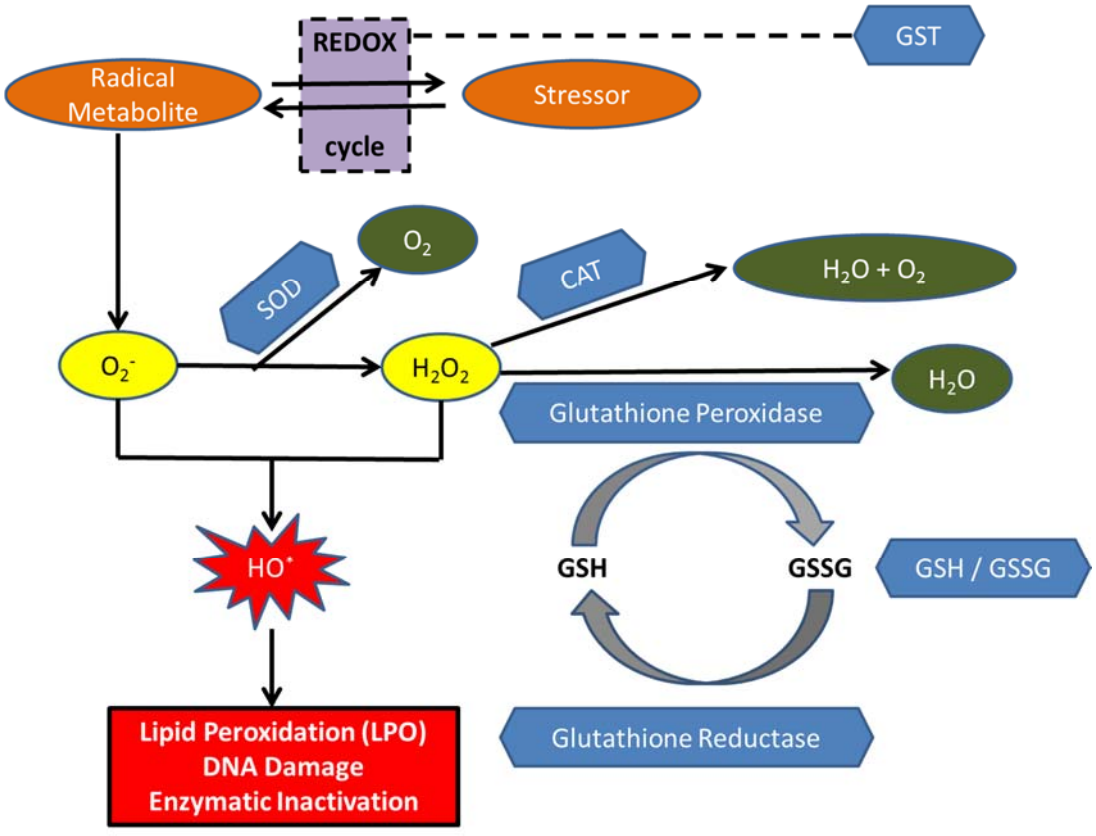

Fig. 1.7 Scheme of oxidative stress generated by reactive oxygen species (ROS).

3.4. Biomarkers at an organism level:

Energy Reserves

Energy reserves (carbohydrates, lipids and proteins) are important in all organisms for their maintenance, growth and reproduction, but under severe conditions of stress they can be mobilized as response and therefore they can be used as biomarker (Huggett et al., 1992). The most quickly mobilized energy reserve in an organism under stress is the glycogen and its depletion has already been observed in several studies (e.g. Huggett et al., 1992; Santos et al., 2011; Santos et al., 2010b). The measurement of glycogen represents a useful measurement of the relative energy status of an organism in time and can predict effects at higher biological organizational levels (Huggett et al., 1992).

As the stress increases in intensity or duration the lipids content is the second energy source to be consumed and although they can provide more energy to the organism than glycogen, they are slowly transformed into energy being this the reason why they respond usually in long-term exposures (Huggett et al., 1992).

The last energy resource to be affected is proteins, which are the dominant constituent fraction of organisms, and under severe conditions can be mobilized by invertebrates to gain energy by the oxidation of amino acids (Bayne, 1973; Giles, 1984). The measurement of proteins as energy source has normally little utility, unless the stress induced by the experimental procedures is extremely high (Huggett et al., 1992).

Cellular Energy Allocation (CEA)

The determination of the cellular energy allocation (CEA) is calculated by converting energy reserves into energetic equivalents using the energy of combustion determined by Gnaiger (1983). The CEA takes into consideration the energy available (E_a) as the sum of carbohydrates, lipids and proteins, and the energy consumption (E_c) estimated by measuring the electron transport activity (ETS) at a mitochondrial level.

The integration of E_a and E_c into an index has then calculated by different ways according to different authors. In a study published by de Coen et al. (1995) the determination of CEA was calculated the integration into time (t) of the E_a and E_c according to the following formula:

$$CEA\,(J/mg\ org) = \frac{(\int_0^t E_a.dt - \int_0^t E_c.dt)}{t}$$

In the study by Verslycke et al. (2004), the determination of CEA is only calculated through the coefficient between Ea and Ec (Ea/Ec) and in a study by the same author (Verslycke et al., 2003) the determination is calculated by the reason between the difference in the available energy and the average energy consumption (ΔEa/Ec). Several other ways of calculating CEA are also presented throughout the literature (e.g. Moolman et al., 2007; Smolders et al., 2004), being all of them based in the available energy and the energy consumption.

4. Nickel and Dimethoate

For the development of these studies two stressors belonging to different chemical groups were used: the metal nickel and the pesticide dimethoate.

Nickel (Ni) is a naturally occurring element in the environment and it is considered an essential trace element for diverse biotic functions in organisms. Due to the high usage in industry, and the involuntary anthropogenic release Ni can reach high concentrations in soils (Phipps et al., 2002).

The information on Ni regarding its effects on soil invertebrates when compared to other metals is still very limited, and it is most focused on its bioaccumulation or in acute testing, by assessing mortality (e.g. Lock and Janssen, 2002; Phipps et al., 2002).

As stated previously, the isopod's hepatopancreas (HP) is the main internal organ for metal handling and its cells pass through a daily cycle of ultrastructural changes in which the contents of the *'B'* cells apical cytoplasm are voided in an apocrine manner over ~11h, which is also a way to expel metals (e.g. Köhler et al., 1996; Tarnawska et al., 2007).

The mechanisms of Ni toxicity are still very poorly understood, and besides the oxidative stress caused by this metal (as all other metals), in studies with cellular lines (Lee et al., 1995), fish (Pane et al., 2003) or daphnids (Vandenbrouck et al., 2009) it is considered a carcinogenic metal, that impacts gene transcription and translation processes and even in the phosphate cycle.

Dimethoate (Fig. 1.8) is a widely used organophosphate insecticide, and one of most used within this group of substances. It has a selective toxicity towards insects, by acting in the enzyme acetylcholinesterase, therefore inhibiting the cleavage of acetylcholine, which will result in extensive cholinergic stimulation and neurotoxicity (de Coen and Janssen, 2003).

Toxicity of dimethoate to invertebrates, specifically for terrestrial isopods has already been studied focusing mainly on mortality, reproduction and behaviour and also on the joint effects of this insecticide with other stressors (e.g. Bayley, 1995; Engenheiro et al., 2005; Fischer et al., 1997; Loureiro et al., 2005; Santos et al., 2011).

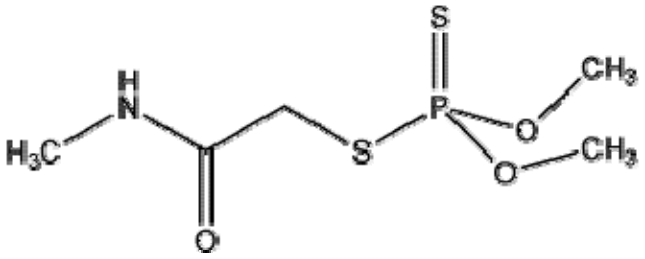

Fig. 1.8 Dimethoate molecular structure.

5. Conceptual framework

The global aim of this thesis was to evaluate and assess the effects of a pesticide (dimethoate) and a metal (nickel), as model chemicals, within different organizational levels, looking at the detoxification pathways (enzymatic biomarkers) and associated energy costs (energy content quantification, energy consumption and CEA), effects at the individual level (mortality), that could be transposed to the population level, and leading to a metabolomic analysis (using ^{1}H-NMR) and finally a gene expression analysis (RT-qPCR and RNA-Seq analysis). In addition, the variation of several abiotic factors such as temperature, soil moisture and UV radiation was also studied. The study on the effects induced by abiotic factors on biomarkers and energy reserves will bridge not only the gap on the knowledge of possible variations within chemical exposures, but also it can be used as guidance for the standardization of isopod's ecotoxicological tests.

The results from this study enable to better understand how these stressors affect the organisms and how isopods dealt with stressors, by trying to return to a "hormetic status". In the general conclusion chapter an integration of all responses is presented and a global view on the detoxification process carried out by this species is proposed. This outcome can be then used in future studies, along with new insights on the reasons why terrestrial isopods can "handle" these two stressors.

In order to achieve the global study goal, several approaches were carried out and are expressed in the present thesis organized into 8 chapters, as described below:

***Chapter I* – General Introduction and objectives.**

***Chapter II* – The effects of temperature, soil moisture and UV radiation in biomarkers and energy reserves of the isopod *Porcellionides pruinosus*.** Using biomarkers and energy reserves as endpoints, the effect of several abiotic factors such as temperature, soil moisture and UV radiation were determined and will serve as support for further studies, supporting also the development of future standardized ecotoxicological assays involving terrestrial isopods. Within the overall work, this study was a base to determine the "optimal conditions" for de exposures performed in the following chapters, in order to observe responses that can

be attributed to the abiotic factors. In addition, these results will be also useful to set up experiments on co-exposures with chemicals.

***Chapter III* – Biomarkers and energy reserves in the isopod *Porcellionides pruinosus*: The effects of long-term exposure to dimethoate.** In order to determine the mechanisms of action of dimethoate within terrestrial isopods, and how they cope with this stressor, dimethoate toxicity was evaluated by using as endpoints biomarkers and energy reserves contents throughout an exposure period of 28 days, followed by a recovery period of 14 days. Within the same scope and considering the effects that may result from a co-exposure of dimethoate and (different) temperature, organisms were exposed to dimethoate at two temperatures: 20°C and 25°C. The concentrations used for dimethoate exposure were based in the recommended appliance field dose and the equivalent to an Ec50 value (from the literature). The results obtained provided general responses that were then investigated in lower organizational levels. The mortality observed in this study could be related to high inhibition levels of the enzyme AChE, but also to oxidative stress and cellular damage. The pathways involved in the processes presented before and the alterations in the energy reserves levels were then investigated in Chapter V.

***Chapter IV* – Long-term exposure of the isopod *Porcellionides pruinosus* to nickel: cost in the energy budget and detoxification enzymes.** Using the same design conditions described in the previous chapter, but only under a single temperature regime (20°C), organisms were exposed to nickel and biomarkers and energy reserves contents were determined as endpoints. The two concentrations used for exposure were the upper limit within the Canadian Framework and 5x that concentration, both environmental relevant concentrations. As in the previous chapter, the results obtained in this study were used as starting point and further explored in Chapter V.

***Chapter V* – Metabolomic responses of the isopod *Porcellionides pruinosus* to nickel and dimethoate exposure assessed by ^{1}H NMR spectroscopy.** To evaluate the toxicity of the two stressors at a metabolomic level, three sampling times (96h, 7 days and 14 days) were chosen for ^{1}H-NMR analysis, and a metabolomic profile of the species *P. pruinosus* was defined along with the metabolite variation within this timeline. The results obtained in this chapter allowed integration and a better understanding of the data from the previous two chapters. For example, in the case of nickel, it was possible to observe that variations in

glutathione-related enzymes involved in handling oxidative stress were inhibited not only by processes of the enzyme-metal interaction previously described in literature, but also by disturbances in the pathways involved in the formation of these enzymes. In the case of the pesticide dimethoate, it was also possible to observe interferences in the pathways involved in neurotransmission, or evidences of induction of moult processes, which were also observed for the metal nickel. The study served also as a bridge to Chapter VII, where up/down regulation in specific genes could then be translated or non-translated into the final proteins.

***Chapter VI* – Tracking of novel potential biomarkers in *Porcellionides pruinosus* (Isopoda) exposed to nickel: a transcriptomic approach.** The work developed in this Chapter can be divided into two parts. In order to investigate the specific genes in the pathways that were determinant and involved in nickel and dimethoate toxicity, it was necessary to develop a deeper study at a molecular level. Here, the transcriptome plays an important role, since without a genome or a transcriptome for terrestrial isopods, it would not be possible to develop primers, essential for the development of the work presented in Chapter VII, so we performed the first full transcriptome of a terrestrial isopod was performed. This transcriptome will not only serve as base for further genomic studies to be developed for terrestrial isopods, but also for evolutionary studies. On the second part of this Chapter the toxicity effects of nickel at a genomic level were evaluated through a RNA-Seq analysis of a 96h exposure at 50mg and 250 mg Ni/kg soil (similarly to the previous chapter). This RNA-Seq analysis allowed to determine pathways that were being impacted by nickel, and also to bring new insight into nickel's mode of action.

***Chapter VII* – Molecular responses of *Porcellionides pruinosus* (Isopoda) when exposed to dimethoate and nickel.** Using a long-term exposure with recovery period, like the one used on Chapter II and III, a total of 11 genes and 4 housekeeping genes were evaluated, all related to pathways that were previously highlighted as potentially impacted by theses stressors. This study brought the information needed at a genomic level, showing genes transcription throughout the exposure and recovery period tested in Chapters III and IV. This last study allowed the construction of an outcome pathway for both nickel and dimethoate present in Chapter VIII.

***Chapter VIII* – General conclusions.**

6. Relevance of the dissertation

The use of terrestrial isopods in ecotoxicology for soil quality evaluation or for the screening of chemical toxicity, such as metals, pesticides, nanoparticles, endocrine disruptors and others, has been already performed, but the elaboration of guidelines/standard tests are still inexistent. Although some of the reason for the lack of theses protocols are related to the high variability of results obtained from isopod exposures, and/or the diurnal hepatopancreas' cells cycle and moult, the new and more detailed information provided by this study may serve as turn point to minimize/overcome the high variability observed at higher organizational levels.

The work presented in this dissertation, regarding the evaluation of two model stressors, a metal and a pesticide, will provide a more detailed information to complement the second point stated before. Among the results obtained, the emphasis on the first metabolic profile of isopods exposed to these two stressors and the first full transcriptome of a terrestrial isopod, can be in the future used as foundations for further studies. The analysis of the biomarkers and energy-related responses to abiotic factors also poses important information for the development of standardized tests.

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CHAPTER II

The effects of temperature, soil moisture and UV radiation in biomarkers and energy reserves of the isopod Porcellionides pruinosus.

The effects of temperature, soil moisture and UV radiation in biomarkers and energy reserves of the isopod *Porcellionides pruinosus*

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Abstract:

Terrestrial isopods from the species *Porcellionides pruinosus* were exposed to different ranges of temperature, soil moisture and doses of UV radiation. For the temperature and soil moisture experiments, organisms were sampled after 48h, 96h and 14 days of exposure, whereas in the UV experiment, they were sampled at the end of the exposure periods, that consisted on a single-pulse with duration ranging from 15 min to 8h. For each sampling time the acetylcholinesterase (AChE), glutathione *S*-transferases (GST) and catalase (CAT) activities were determined, as well as lipid peroxidation rate (LPO). Energy content (lipids, carbohydrates, proteins) and energy related parameters: energy available (Ea), energy consumption (Ec) and cellular energy allocation (CEA) were also determined, along with mortality.

The results obtained showed that increases in temperature will affect life traits and specific strategies for isopods to manage their energy budget, in order to handle oxidative stress. It also showed that this species is acclimated to lower moisture scenarios, whereas in case of flood scenarios the turnover point between optimal conditions and mortality is very narrow, which may lead to the local extinction of populations in specific micro-habitats. This study also showed that UV-R also poses an important stressor for isopods that should be taken in consideration, as the actual doses nowadays present significant negative impact in these organisms.

The study also emphasises that the effects of abiotic factors should be included and take into consideration by policymakers and that the inclusion of abiotic effects in ecotoxicological tests should be included in the analysis of any stressor to improve chemical risk assessment.

Keywords**:** climate changes, abiotic factors, energy related parameters, oxidative stress, neurotoxicity.

1. Introduction

Organisms are constantly adapting their physiology and life traits to their habitat characteristics. According to the report "Climate Change 2013: The Physical Science Basis" by the Intergovernmental Panel on Climate Change, the climate is changing, global average air and ocean temperatures are increasing, extreme weather conditions are being observed more often (including droughts and heavy precipitation), the ultraviolet radiation (UV-R) reaching the planet is higher due to the depletion of ozone layer and even the pH of water and soil is being affected (IPCC, 2013).

The changes occurring in the ecosystems constitute an important concern to the scientific community, not only due to the already described negative effects, but also because these effects are gradual and hard to detect, leading to questions like "how are these gradual changes affecting organisms?", "will organisms in a specific ecosystem deal with the stressors by moving to a more suitable one, will they adapt or will this changes tend to increase the risk of local extinction?", "how are organisms coping with these stressors?" or "will organisms be able to get to a new homeostasis status?".

Trying to answer the previous questions, we focused our attention on the effects of climate changes in terrestrial ecosystems and more specifically on the soil compartment, an important and very complex environment, not only due to its usages, but also because of its structure, function, taxonomic diversity and trophic interactions. In addition, the impact in this compartment will also affect social and economic human activities and therefore human health (Wild, 1993).

For that, terrestrial isopods (*Porcellionides pruinosus*) were selected as test-species, due to their important role on decomposition and fragmentation processes, which may then reflect the effect of stressors in the overall soil functions, causing changes on soil quality and soil services (Drobne, 1997; Lokke and vanGestel, 1998; MEA, 2005). To understand how isopods are being affected by abiotic factors, traditional traits like survival, reproduction or feeding rates, may not entirely reflect the effects of these stressors, since they might not detect early effects. For this reason, the endpoints chosen to evaluate effects caused by abiotic changes were enzymatic and physiological biomarkers, along with energy related parameters. This choice for biomarkers is mainly due to their sensitivity, quickness and accurate relationship between the stressor exposure and the respective biological response (Morgan et al., 1999). Regarding the measurement of energy related paremeters, they are essencial for the organisms lifetraits such as growth, reproduction, maturation or maintaince, and their depletion affects negatively the population dynamics and structures (de Coen and Janssen, 2003). The measurement of energy reserves as an endpoint has already been used sucessfully in several previous works (e.g. Donker, 1992; Morgado et al., 2013; Stanek et al., 2006), using terrestrial isopods exposed to chemical or abiotic stressors.

Therefore, the main goals of the present study was to simulate changes in abiotic factors that can occur in the soil compartment and determine their effects on biomarkers and energy reserves of the terrestrial isopod *Porcellionides pruinosus*. The abiotic factors used were temperature (increase and decrease from the optimum), soil moisture (simulating drought and flood situations), and exposure to an increase dose of UV-R.

The results obtained here will help understanding the processes that terrestrial isopods undergo when exposed to these stressors, how they are coping with them, the pathways that will be activated by the stressors and how the energy budget will be affected.

2. Materials and methods

2.1. Test Organism and Culture Procedure

The organisms used in this study belong to the species *Porcellionides pruinosus* Brandt (1833), and were previously collected from a horse manure heap and maintained for several generations in laboratory cultures. In culture, isopods were fed *ad libitum* with alder leaves (*Alnus glutinosa*) and maintained at 22 ± 1°C, with a 16:8 h (light:dark) photoperiod. Twice a week cultures were water sprayed and food was provided. Only adult organisms (15-25 mg wet weight) were used in these experiments and no distinction between sexes was made, although pregnant females were excluded from trials. Organisms with abnormalities or under moulting were also excluded.

2.2. Exposures

Tests were performed in plastic boxes (14.3 length x 9.3 width x 4.7 height cm), containing approx. 2 cm LUFA 2.2 soil with 12 isopods per test-box. The properties of this soil included a pH = 5.5 ± 0.2 (0.01 M CaCl2), water holding capacity = 41.8 ± 3.0 (g/100 g), organic C = 1.77 ± 0.2 (%), nitrogen = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand. Organisms were collected from culture boxes, weighted (15-25mg) and placed in each test-box with alder leaf disks (Ø 10 mm, ± 20 mg) supplied as food. A total of five replicates were used for each exposure scenario and each replicate corresponds to one box with 12 isopods.

The exposure lasted for 14 days and biomarkers and energy related parameters were measured after 48 h, 96 h and at the end of the exposure period.

2.2.1. Temperature exposure

Tests were performed in LUFA 2.2 soil with a final moisture content equivalent to 50% WHC. Test-boxes were then placed at different temperatures in climatic chambers: 10ºC, 15ºC, 20ºC (used as control temperature), 25ºC and 30ºC, with a 16:8 h (light:dark) photoperiod and checked on a daily bases to maintain soil moisture.

2.2.2. Soil moisture exposure

In this exposure trial, soil was adjusted for moisture content with 20%, 40%, 60% (used as control) and 80% WHC; exposures were carried out under a 16:8 h (light:dark) photoperiod regime and checked on a daily bases to maintain soil moisture, using a weight differential approach.

2.2.3. UV irradiance exposure

UV irradiance was supplied by an UV lamp (Spectraline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 313 and 365 nm corresponding to UV-B and UV-A, respectively) that was placed 30 cm above the boxes containing the isopods. In order to filter UV-C wavelengths, lamp was covered with a clear cellulose acetate sheet (0.003 mm, Grafix plastics, USA) that had been previously irradiated during 8h to allow radiation intensity stabilization. All the parameters regarding the exposure are presented in Table 2.1. Total radiation intensity was measured by using a spectro-radiometer connected to a monochromator and analysed with BenWin+ software (Bentham Instruments, Reading, UK).

Total biologically effective doses of UV-R (equation 1) used on the exposures were calculated, using the total UV intensity and integrated into time to obtain the UV dose (equation 2). Afterwards the CIE reference action spectrum for both the erythema in human skin (McKinlay, 1987), and the reference action spectrum for DNA damage (Setlow, 1974)

correction values were used to present the final UV-R dose. The erythema in human skin UV dose was calculated using intensities between 280 and 400nm and for DNA damage between 280 and 364 nm. The UV doses were also related with locations considered ecologically relevant in which similar values were observed (KMNI, 2013 - Table 2.1).

$$UV\ (\text{J. cm}^{-2}) = 10 \text{ x } UV_{intensity}\ (\text{mW. cm}^{-2}) \text{ x } time\ of\ exposure\ (\text{s}) \qquad \text{(equation 1)}$$

$$[UV\ dose]_0^n\ (\text{J. m}^{-2}) = \frac{UV_0\ (\text{mW. cm}^{-2}) - UV_n\ (\text{mW. cm}^{-2})}{2} + UV_n\ (\text{mW. cm}^{-2}) \qquad \text{(equation 2)}$$

Table 2.1 Exposure UV doses, after correction for DNA damage (Setlow, 1974) and human skin erythema (McKinlay, 1987) with the corresponding exposure time and peak intensity. Locations where a similar average erythemal UV dose were observed are also presented for ecological relevance (KNMI, 2013). (a) - between December and February, (b) - between March and May, (c) - between June and August, (d) - between September and November. *- Higher UV doses whose correspondence was not found in the literature.

Exposure time (min)	Average peak intensity ($mW.m^{-2}$)		UV dose (corrected for DNA damage) ($kJ.m^{-2}$)	UV dose (corrected for Human skin erythema) ($kJ.m^{-2}$)	Location where dose is considered ecologically relevant
	313 nm	365 nm			
15	113.39	44.11	0.85	0.47	Kingston (Australia)[c]
30	107.21	43.85	1.55	0.86	Sodankyla (Finland)[b]
60	100.45	44.09	2.77	1.66	Angra do Heroísmo (Azores, Portugal)[d]
90	99.78	43.56	4.22	2.48	Sodankyla (Finland)[c]
120	100.49	44.67	5.36	2.98	Brisbane (Australia)[b]
180	97.41	43.70	8.41	4.64	Izana (Tenerife, Spain)[c]
240	103.40	45.10	12.03	6.62	Alice Springs (Australia)[a]
300	105.48	45.25	14.99	8.32	*
360	91.43	42.98	15.34	8.48	
480	116.97	42.35	27.97	15.42	

2.3. Biomarkers and energy related parameters

The protocol used to process samples was previously described by Ferreira et al. (2010) and is thoroughly described in the supplementary data. Pools of two organisms were used as a replicate for the determination of all biomarkers, except for AChE. For AChE a single organism was divided into head and body, and each individual head was used as a replicate. For the energy related parameters, only one organism was used which corresponded to one replicate. The lipid peroxidation (LPO) determination was previously described by Bird and Draper (1984) and Ohkawa et al. (1979) and further adapted to microplate. The glutathione *S*-transferases (GST) and glutathione peroxidase (GPx) activities were determined as described by Habig et al. (1974) and Mohandas et al. (1984), respectively. Catalase (CAT) activity was determined based on the method described by Clairborne (1985) and adapted to microplate. The acetylcholinesterase (AChE) activity was assessed according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). For all biomarkers, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-test set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

To determine total protein, carbohydrate and lipid contents, energy consumption (Ec) and energy available (Ea) and cellular energy allocation (CEA), protocols were adapted from de Coen and Janssen (1997). The Ea, Ec and CEA value were calculated as described by Verslycke et al. (2004):

Ea (available energy) = carbohydrates + lipids + proteins (mJ/mg org.)
Ec (energy consumption) = ETS activity (mJ/mg org. /h)
CEA (cellular energy allocation) = Ea/Ec (h)

2.4. Data Analysis

A one-way analysis of variance (ANOVA) was performed to compare differences between treatments at each sampling time followed by a Dunnett's comparison test to discriminate statistically different treatments from the control (SPSS 1999). When possible, data

transformation was used to achieve normality. When data did not show a normal distribution or homoscedasticity, the non-parametric test Kruskal-Wallis One Way Analysis of Variance on Ranks was used, followed by a Dunn's test.

Data values that were higher or lower than the mean value, plus or minus two times the standard deviation, were considered outliers, and withdrawn from analysis (Rousseeuw et al. 1993). A two-way analysis of variance (two-way ANOVA) was performed to check for interactions between time and treatments. The one-way ANOVA and two-way ANOVA used $\alpha = 0.05$ for significance.

3. Results

3.1. Temperature exposure:

Mortality was only observed after 14 days of exposure at 30ºC but still below 10%.

Before exposure (T0), the mean value for AChE activity was 58.77 ± 3.44 U/mg protein (± SE), and no significant differences were observed between T0 and the further sampling times at 20ºC. A significant increase was only observed for the temperature of 30ºC after 96 h of exposure (One Way ANOVA, *ln* transformation, $F_{4,19} = 5.904$; Dunnett's test, $p = 0.003$ - Fig. 2.1). When applying a two-way ANOVA, there was a significant interaction between temperature and time of exposure (Two Way ANOVA, $F_{12,75} = 2.557$; $p = 0.007$).

For the catalase activity (Fig. 2.1) significant differences from the 20ºC exposure were only observed again for the temperature of 30ºC with decreases after 48 h and 14 days of exposure (One Way ANOVA $F_{4,17} = 8.614$; Dunnett's test $p < 0.001$ and $F_{4,19} = 13.988$; Dunnett's test $p < 0.001$ respectively). A two-way ANOVA revealed no significant interaction between temperature of exposure and time (Two Way ANOVA, $F_{8,55} = 0.825$; $p = 0.584$). Before exposure, organisms showed a mean value for CAT of 7.58 ± 0.41 U/mg protein (± SE). Differences between T0 and the sampling times were observed at the extreme temperatures used: 15ºC after 48 h of exposure (One Way ANOVA, $F_{3,16} = 4.096$; Dunnett's test $p = 0.025$), for 30ºC after 48 h and 14 days of exposure (Kruskal-Wallis, $H = 13.377$; d.f. = 3;

Dunn's test $p = 0.004$) and within the control temperature: 20ºC after 48 h and 96 h (One Way ANOVA, $F_{3,16} = 4.186$, Dunnett's test $p = 0.024$).

The activity of the biomarker glutathione *S*-transferases (Fig. 2.1) showed a significant increase after 96 h of exposure at 25ºC (One Way ANOVA, $F_{4,20} = 5.171$; Dunnett's test $p = 0.005$) and after 14 days of exposure at 10ºC (One Way ANOVA, $F_{4,20} = 14.560$; Dunnett's test $p < 0.001$) when compared to the 20ºC exposure. After 14 days of exposure opposite trends were observed at 25ºC and 30ºC with a significant decrease in GST activity (One Way ANOVA, $F_{4,20} = 14.560$; Dunnett's test $p < 0.001$). The two-way ANOVA showed a significant interaction between the temperature of exposure and time (Two Way ANOVA, $F_{8,60} = 4.721$; Dunnett's test $p < 0.001$). The mean value for GST activity at T0 was 148.96 ± 2.65 U/mg protein (± SE). Significant differences between T0 and the sampling times were observed at 30ºC for 96 h and 14 days of exposure (One Way ANOVA, $F_{3,16} = 3.631$; Dunnett's test $p = 0.036$).

The lipid peroxidation rate (Fig. 2.1) did not show any significant difference between the 20ºC and all the other temperature used for exposure in all sampling times, although the two-way ANOVA showed a significant interaction between temperature of exposure and time (Two Way ANOVA, $F_{8,58} = 4.361$; Dunnett's test $p < 0.001$). The mean value for LPO rate at T0 was 50.03 ± 0.88 mmol TBARS/g org (± SE) and significant differences between T0 and the sampling times were observed at 15ºC and 20ºC for 48 h and 96 h of exposure (One Way ANOVA, $F_{3,15} = 7.140$; Dunnett's test $p = 0.003$; One Way ANOVA, $F_{3,16} = 6.102$; Dunnett's test $p = 0.006$), at 25ºC for 48h and 14 days of exposure (One Way ANOVA, $F_{3,15} = 8.292$; Dunnett's test $p = 0.002$) and at 30ºC for all sampling times (One Way ANOVA, $F_{3,16} = 7.986$; Dunnett's test $p = 0.002$).

The statistical analysis of glutathione peroxidase (Fig. 2.1) showed a significant increase only for 15ºC after 48 h of exposure when compared to the 20ºC regime (One Way ANOVA, $F_{4,17} = 3.304$; Dunnett's test $p = 0.036$); in addition the two-way ANOVA also showed a significant interaction between the temperature of exposure and time. The mean activity for GPx before exposure was 1.23 ± 0.11 U/mg protein (± SE). Significant differences between T0 and the sampling times were observed also for 15ºC after 48 h of exposure (One Way ANOVA, $F_{3,15} = 4.208$; Dunnett's test $p = 0.024$).

When analysing the energy reserve content (Fig. 2.2), no significant differences were observed for carbohydrates, lipids and proteins, for all sampling times between all temperature regimes used and the control temperature of 20ºC. Also the two-way ANOVA did not show any significant differences between the temperature and the time of exposure for all of the energy reserves content. The mean values at T0 were 555.11 ± 34.95 mJ/mg org for lipids, 218.43 ± 4.79 mJ/mg org for carbohydrates and 548.89 ± 20.48 mJ/mg org for proteins (± SE). Significant differences between T0 and sampling times were observed for carbohydrates at 15ºC after 14 days of exposure (One Way ANOVA, $F_{3,16} = 4.058$; Dunnett's test $p = 0.025$), at 20ºC at all sampling times (One Way ANOVA, $F_{3,16} = 9.989$; Dunnett's test $p < 0.001$) and at 25ºC after 96 h and 14 days of exposure (One Way ANOVA, $F_{3,16} = 7.066$; Dunnett's test $p = 0.003$). For proteins, significant differences between T0 and sampling times were observed only at 15ºC after 48h and 14 days of exposure (One Way ANOVA, $F_{3,16} = 3.845$; Dunnett's test $p = 0.030$), and no significant differences were observed for lipids.

For the energy related parameters (Fig. 2.2), no significant differences between temperature regimes and 20ºC were observed for Ea, but for Ec significant decreases were observed after 48h of exposure at 25ºC and 30ºC (Kruskal-Wallis, $H = 16.542$; d.f. = 4; Dunn's test $p = 0.002$). The CEA rate showed a significant increase after 48 h of exposure for 25ºC and 30ºC when compared to the 20ºC (One Way ANOVA, *ln* transformation, $F_{4,20} = 15.843$; Dunnett's test $p < 0.001$) and significantly decreasing after 96 h of exposure for 10ºC and 25ºC (One Way ANOVA, $F_{4,19} = 4.858$; Dunnett's test $p = 0.007$). The two-way ANOVA showed no significant interaction between temperature of exposure and time for Ea (Two Way ANOVA, $F_{12,80} = 1.427$; $p = 0.171$), but for Ec (Two Way ANOVA, $F_{12,80} = 3.099$; $p = 0.001$) and CEA (Two Way ANOVA, *ln* transformation, $F_{12,78} = 4.378$; $p < 0.001$) significant interactions were observed. The mean values at T0 were 1322.43 ± 49.34 mJ/mg org for Ea, 0.36 ± 0.01 mJ/min/mg org for Ec and 61.53 ± 2.84 for CEA (± SE). Significant differences between T0 and sampling times were observed for all temperatures and sampling times except for the Ea of isopods exposed at 15ºC for 48 h. The Ec significantly decreased in all sampling times and temperatures of exposure ($p < 0.01$), and for CEA significant increases were observed for all temperatures and sampling times ($p < 0.01$), except for 20ºC after 48h of exposure.

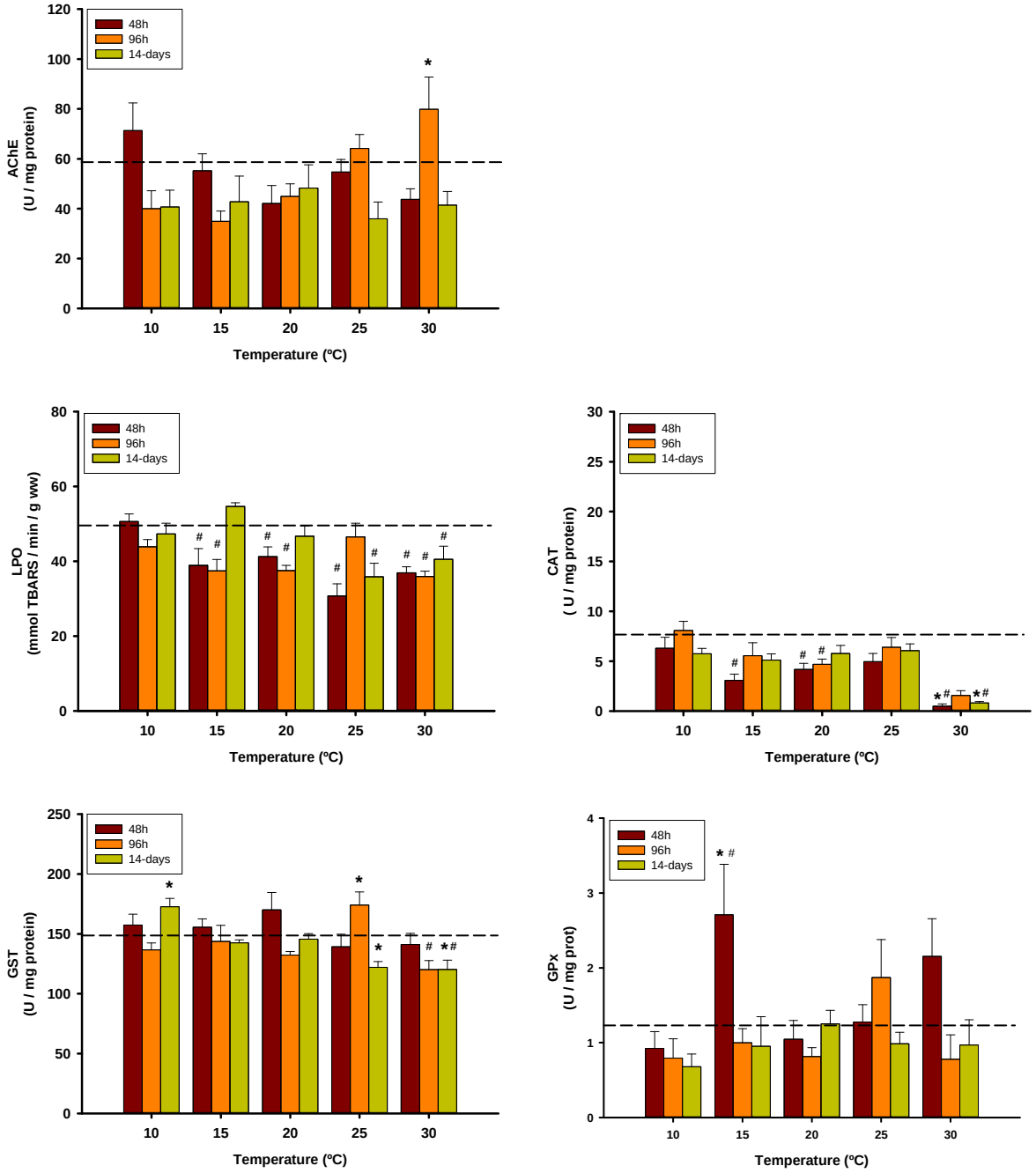

Fig. 2.1 Activity of the biomarkers acetylcholinesterase (AChE), catalase (CAT), glutathione *S*-transferases (GST), lipid peroxidation (LPO) and glutathione peroxidase (GPx) of *Porcellionides pruinosus* exposed to different temperatures and sampled after 48h, 96h and 14 days of exposure. *- denotes significant difference when compared to the same sampling time at 20ºC (control); #- denotes significant difference when compared to the T0 (before exposure); dashed line represents mean activity at T0 (before exposure).

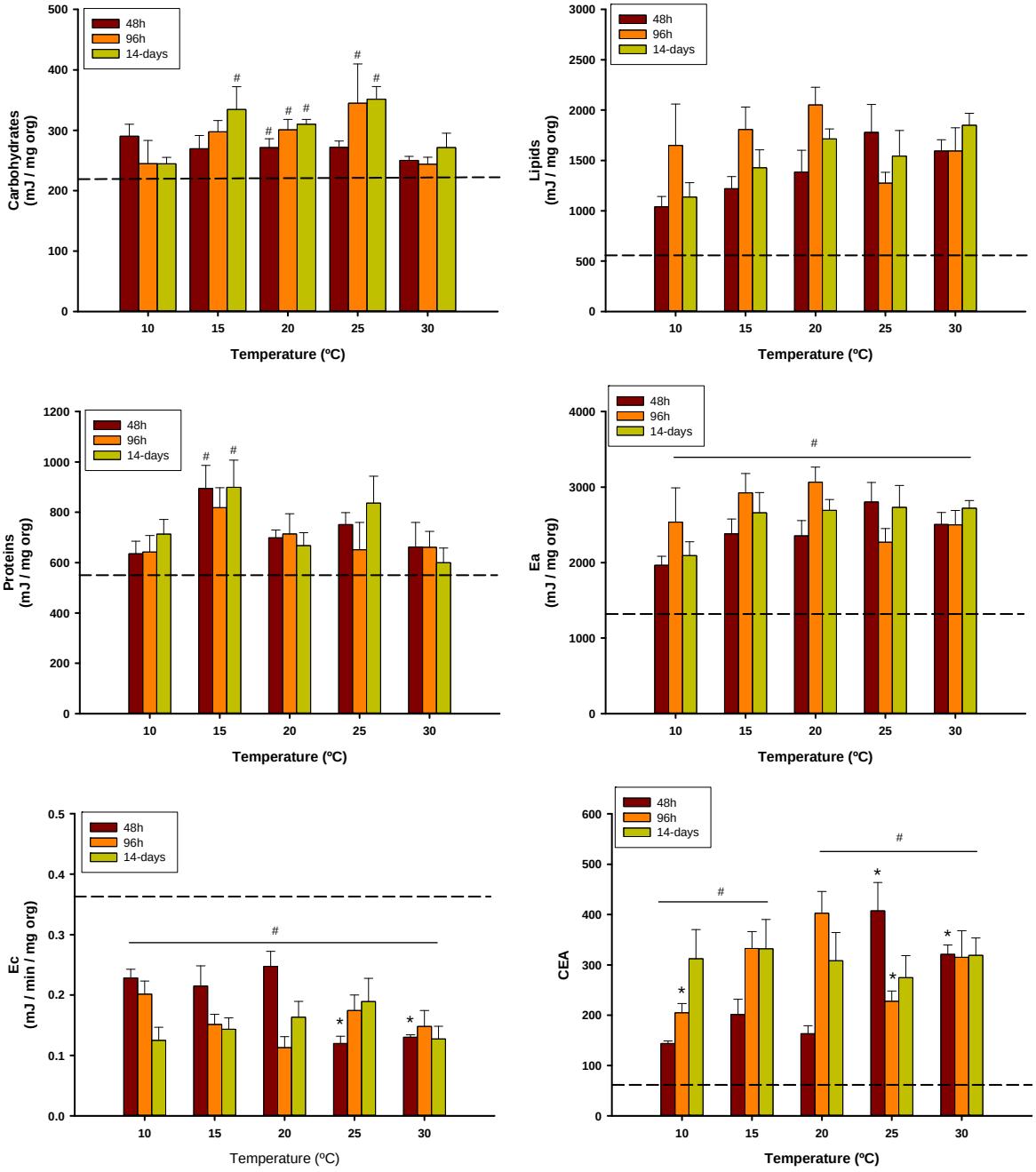

Fig. 2.2 Energy related parameters: lipids, carbohydrates and proteins content, and the balance for the energy available (Ea), energy consumption (Ec) and cellular energy allocation (CEA) of *Porcellionides pruinosus* exposed to different temperatures and sampled after 48h, 96h and 14 days of exposure. *- denotes significant difference when compared to the same sampling time at 20ºC (control); #- denotes significant difference when compared to the T0 (time 0); dashed line represents mean value at T0.

3.2. Soil moisture exposure

Considering that organisms were sampled at 48 h, 96 h and at the end of the experiments (14 days), and survival was checked at each time sampled, at 20% WHC two dead organisms were observed at 96 h and one at the 14th day of sampling. At 40% and 60% WHC only one organism died in each treatment. The 80% WHC treatment showed higher mortality after 14 days of exposure, where six organisms were found dead, thus no sampling was performed at that sampling time for the acetylcholinesterase measurement and the energy related parameters, only allowing that three replicates were available for all the other biomarkers (GST, CAT, LPO and GPx). The daily mean variation of water was 0.31% ± 0.08 WHC.

For the analysis of the acetylcholinesterase activity (Fig. 2.3) no significant differences were observed for all sampling times and between the different percentages of moisture when compared to the 60% WHC. The mean value for the AChE activity before exposure (T0) was 68.97 ± 1.10 U/mg protein (± SE). No significant differences between T0 and the sampling times were observed at all soil moisture treatments and sampling times.

The catalase activity (Fig. 2.3) showed no significant differences between the 60% WHC and all other soil moistures tested at all sampling times. The two-way ANOVA analysis did not show any significant interactions between the percentage of WHC and the time of exposure (Two Way ANOVA, *ln* transformation, $F_{9,52} = 1.208$; $p = 0.310$). The mean value for CAT activity before exposure (T0) was 15.86 ± 0.74 U/mg protein (± SE). Significant differences between T0 and the sampling times were observed only for the 40% WHC after 14 days of exposure (One Way ANOVA, *ln* transformation, $F_{3,17} = 12.772$; Dunnett's test $p < 0.001$).

The activity of the biomarker glutathione *S*-transferases (Fig. 2.3) did not show any significant differences between the 60% WHC and all WHC percentages for all sampling times. The two-way ANOVA analysis could not be performed due to the lack of normality. The mean value for the GST activity before exposure (T0) was 161.57 ± 1.91 U/mg protein (± SE). No significant differences between T0 and the sampling times were observed for this biomarker.

The lipid peroxidation rate (Fig. 2.3) did not show any significant difference between the 60% WHC and all the other percentages of WHC for all sampling times. As for the two-way ANOVA analysis, no significant interaction between temperature of exposure and time were observed (Two Way ANOVA, $F_{9,58} = 1.941$; $p = 0.064$). The mean value for the LPO rate before exposure (T0) was 52.73 ± 0.86 mmol TBARS/min/g ww (± SE). Significant differences between T0 and the sampling times were observed for the 20% WHC exposure (One Way ANOVA, $F_{3,17} = 5.376$; Dunnett's test $p = 0.011$), 40% WHC (One Way ANOVA, $F_{3,18} = 3.937$; Dunnett's test $p = 0.030$) and 80% WHC (One Way ANOVA, *ln* transformation $F_{3,17} = 8.412$; Dunnett's test $p = 0.002$) after 14 days of exposure.

The statistical analysis of glutathione peroxidase (Fig. 2.3) showed no significant differences between any sampling time and any percentage of WHC. As for the two-way ANOVA analysis, no significant interaction between temperature of exposure and time were observed (Two Way ANOVA, *exponential transformation*, $F_{9,55} = 1.857$; $p = 0.078$). The mean value for the GPx activity at T0 was 1.23 ± 0.11 U/mg protein (± SE). Significant differences between T0 and the sampling times were only observed for the 20% WHC after 96 h of exposure (One Way ANOVA, $F_{3,17} = 3.538$; Dunnett's test $p = 0.043$)

When analysing the energy reserves content (Fig. 2.4) a significant decrease in lipids content was observed after 48 h of exposure for the 20% and 40% soil WHC when compared to the 60% WHC (One Way ANOVA, $F_{3,16} = 7.857$; Dunnett's test $p = 0.002$) and after 96 h of exposure at 20% soil WHC (One Way ANOVA, $F_{3,16} = 4.662$; Dunnett's test $p = 0.016$). Regarding the time of exposure when compared to the T0, a significant decrease was observed at 20% soil WHC after 96 h of exposure (One Way ANOVA, $F_{3,16} = 6.000$; Dunnett's test $p = 0.006$), a significant increase at 40% soil WHC after 14 days of exposure (One Way ANOVA, $F_{3,15} = 7.489$; $p = 0.003$) and at 80% WHC after 48 h of exposure (One Way ANOVA, $F_{2,12} = 4.549$; Dunnett's test $p = 0.034$). In the case of carbohydrates, significant increases were only observed for the 40% soil WHC after 48 h of exposure when compared to the 60% WHC (One Way ANOVA, $F_{4,20} = 6.542$; Dunnett's test $p = 0.002$). Finally, the protein content did not show any statistical difference within all soil WHC ranges or throughout the time of exposure when compared to the 60% WHC. The mean values for energy reserves at T0 were 94.82 ± 11.17 mJ/mg org for carbohydrates, 2978.46 ± 53.98 mJ/mg org for lipids, 488.29 ± 43.10 mJ/mg org for proteins (± SE).

The analysis of the total available energy (Ea) showed significant decreases between the 60% WHC and the 20% soil WHC after 48 h (One Way ANOVA, $F_{3,15} = 6.604$; Dunnett's test $p = 0.005$) and after 96 h of exposure (One Way ANOVA, $F_{3,16} = 5.250$; Dunnett's test $p = 0.010$). The analysis through time of exposure showed only a significant decrease at the 20% WHC after 96 h of exposure (One Way ANOVA, $F_{3,15} = 6.366$; Dunnett's test $p = 0.005$). As for the energy consumption (Ec), significant decreases were observed at 20% and 80% WHC when compared to the 60% WHC after 48 h of exposure (One Way ANOVA, $F_{3,16} = 6.094$; Dunnett's test $p = 0.006$). The time analysis showed significant decreases at 20% WHC after 96 h of exposure (One Way ANOVA, $F_{3,16} = 7.609$; Dunnett's test $p = 0.002$), at 60% WHC after 48 h and 14 days of exposure (One Way ANOVA, $F_{3,16} = 5.178$; Dunnett's test $p = 0.011$) and at 80% WHC after 14 days of exposure (One Way ANOVA, $F_{2,12} = 12.427$; Dunnett's test $p = 0.001$). The CEA index showed a significant decrease at the 20% WHC after 48 h of exposure (One Way ANOVA, $F_{3,16} = 4.467$; Dunnett's test $p = 0.023$) and a significant increase at 80% WHC after 96 h of exposure (One Way ANOVA, $F_{3,19} = 5.745$; Dunnett's test $p = 0.007$) when compared to the 60% WHC. Mean values for energy related parameters at T0 were 3561.57 ± 91.50 mJ/mg org for Ea, 0.49 ± 0.03 mJ/min/mg org for Ec and 126.80 ± 4.77 for CEA (± SE). Significant differences between T0 and the WHC treatments for Ea were observed only at 20% WHC after 96 h of exposure (One Way ANOVA, $F_{3,18} = 6.366$; Dunnett's test $p = 0.005$). For Ec significant differences were observed at 20% WHC after 96 h of exposure (One Way ANOVA, $F_{3,19} = 7.609$; Dunnett's test $p = 0.002$), 60% WHC after 48 h and 14 days of exposure (One Way ANOVA, $F_{3,19} = 5.178$; Dunnett's test $p = 0.011$) and at 80% WHC after 96 h of exposure (One Way ANOVA, $F_{2,14} = 12.427$; Dunnett's test $p = 0.001$). Finally for CEA, significant increases were observed at 40% WHC after 14 days of exposure (One Way ANOVA, $F_{3,17} = 3.904$; Dunnett's test $p = 0.032$), at 60% WHC after 48 h and 14 days of exposure (One Way ANOVA, $F_{3,18} = 5.536$; Dunnett's test $p = 0.009$) and at 80% after 96 h of exposure (Kruskal-Wallis, $H = 10.500$; d.f. = 2; Dunn's test $p = 0.005$).

Due to the lack of samples at the end of the test for the 80% WHC treatment, the two-way analysis of variance could not be performed for the energy reserves content or energy related parameters.

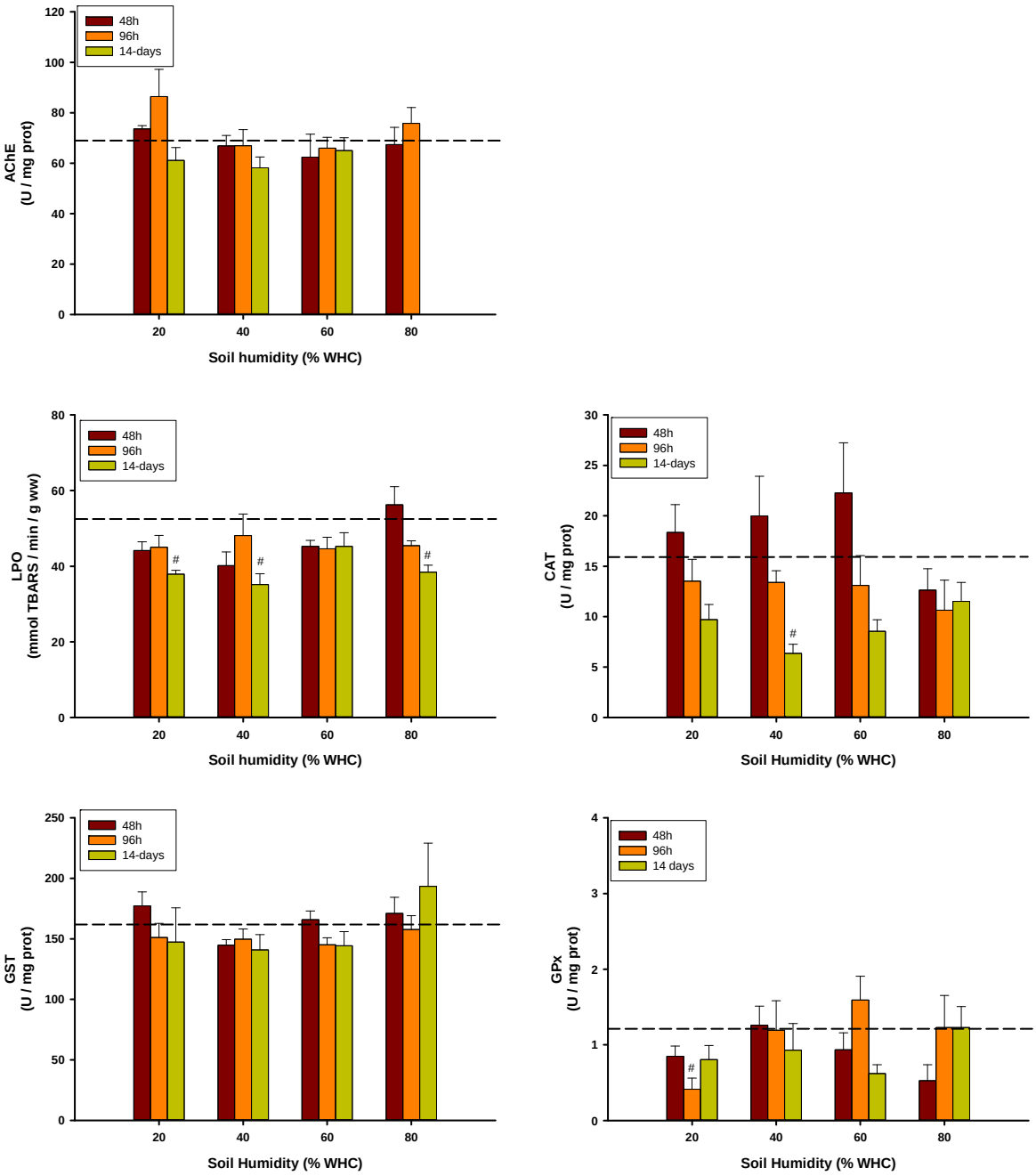

Fig. 2.3 Activity of the biomarkers acetylcholinesterase (AChE), catalase (CAT), glutathione *S*-transferases (GST), lipid peroxidation (LPO) and glutathione peroxidase (GPx) of *Porcellionides pruinosus* exposed to different soil moistures (expressed as water holding capacity- WHC) and sampled after 48 h, 96 h and 14 days of exposure. The lack of data for AChE activity at 80% WHC after 14 days of exposure was due to the high mortality observed. *- denotes significant difference when compared to the related sampling time in the control (60% WHC); $^{\#}$- denotes significant difference when compared to the T0 (time 0); dashed line represents mean activity at T0.

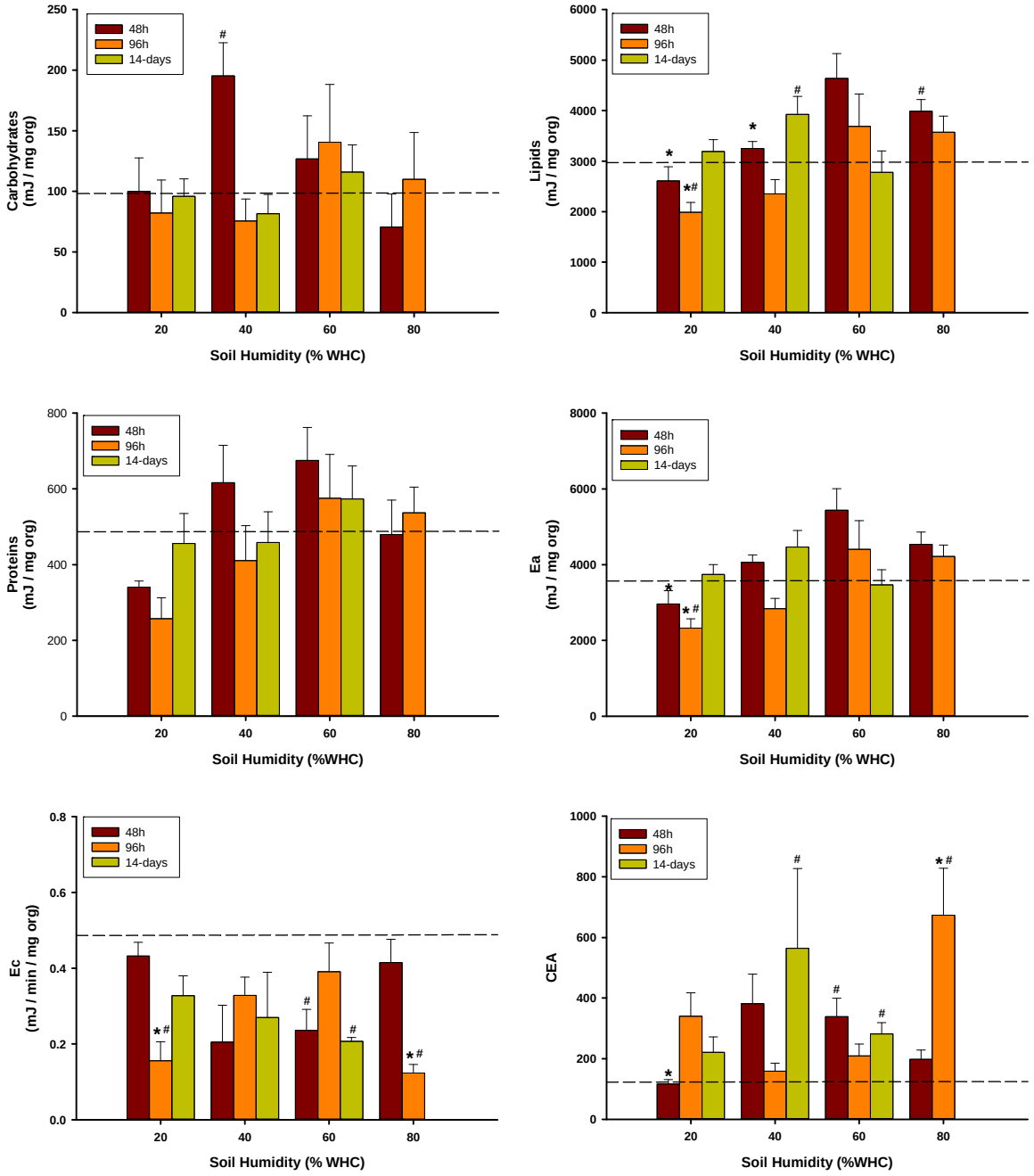

Fig. 2.4 Energy related parameters: lipids, carbohydrates and proteins content, energy available (Ea), energy consumption (Ec) and cellular energy allocation (CEA) of *Porcellionides pruinosus* exposed to different ranges of soil different soil moistures (expressed as water holding capacity- WHC) and sampled after 48 h, 96 h and 14 days of exposure. The lack for 80% WHC after 14 days of exposure is due to high mortality observed.
*- denotes significant difference when compared to the related sampling time in the control (60% WHC);
#- denotes significant difference when compared to the T0 (time 0); dashed line represents mean activity at T0.

3.3. UV irradiance exposure

During the UV irradiance exposure no mortality was observed in any exposure period. For the analysis of the acetylcholinesterase activity, an inhibition was observed until 8.41kJ.m^{-2} of exposure, and afterwards a pattern of increase was observed. When compared to the unexposed organisms, a significant inhibition was observed at 4.22, 5.36 and 8.41 kJ.m^{-2} of exposure, followed by a significant increase at 15.34 and 27.97 kJ.m^{-2} of exposure to values almost twice of those from the control (One Way ANOVA, $F_{10,44} = 27.586$; $p < 0.001$).

Regarding the oxidative stress related biomarkers (Fig. 2.5), no significant differences were observed between the control and any other exposure periods, although CAT appeared to have an inhibition pattern within the exposure to 4.22 kJ.m^{-2}. The activity of the glutathione *S*-transferases showed a significant induction after 1h of exposure (One Way ANOVA, $F_{10,44}= 5.315$; $p < 0.001$), followed by an inhibition pattern. The lipid peroxidation rate only showed a significant decrease at 27.97 kJ.m^{-2} of irradiation (Kruskal-Wallis, $H_{10} = 24.401$; $p = 0.007$). Due to the very low activity found for GPx activity (in some treatments the full set of replicates was similar to the one observed for the blank test) this enzyme was not included.

When analysing the energy reserves content (Fig. 2.6), a significant increase in lipids content was observed in all sampling times after exposure to 12.03 kJ.m^{-2} (One Way ANOVA, $F_{10,44}= 4.499$; $p < 0.001$). In the case of carbohydrates, only a significant decrease was observed at 12.03 kJ.m^{-2} of exposure (One Way ANOVA, ln transformation, $F_{10,42} = 2.876$; $p = 0.008$). Finally for the protein content, no significant differences were observed for all the exposure period (One Way ANOVA, $F_{10,42} = 3.126$; $p = 0.446$). The sum of all the energy content (energy available – Ea) showed significant decreases at 0.85, 2.77, 4.22 and 12.03 kJ.m^{-2} of exposure (One Way ANOVA, $F_{10,42} = 3.914$; $p < 0.001$). As for the energy consumption, no significant differences were observed for all the exposures (One Way ANOVA, $F_{10,44} = 2.222$; $p = 0.034$). The CEA index showed no significant differences between the control and the remaining treatments (One Way ANOVA, *ln* transformation $F_{10,41} = 1.686$; $p = 0.117$).

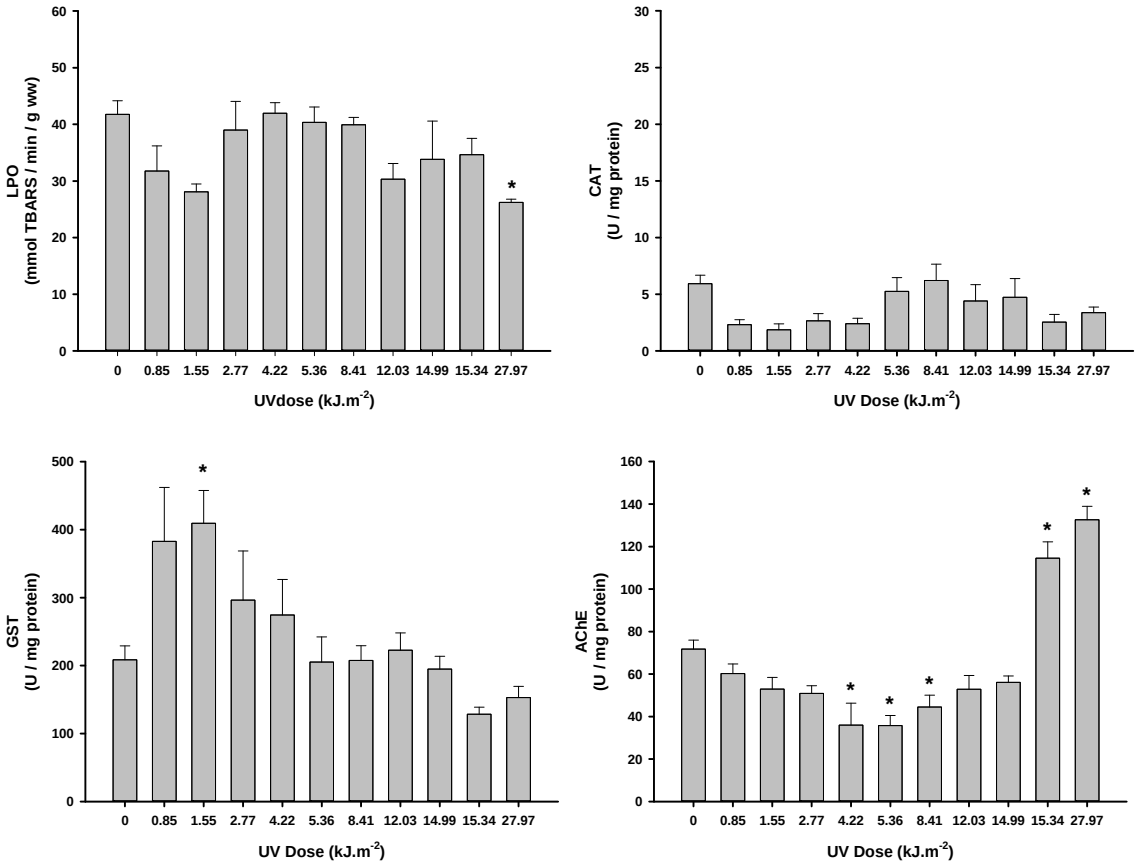

Fig. 2.5 Activity of the biomarkers acetylcholinesterase (AChE), catalase (CAT), glutathione *S*-transferases (GST) and lipid peroxidation (LPO) of *Porcellionides pruinosus* exposed to different doses of UV (dose corrected for DNA damage). *- denotes significant difference when compared to the control.

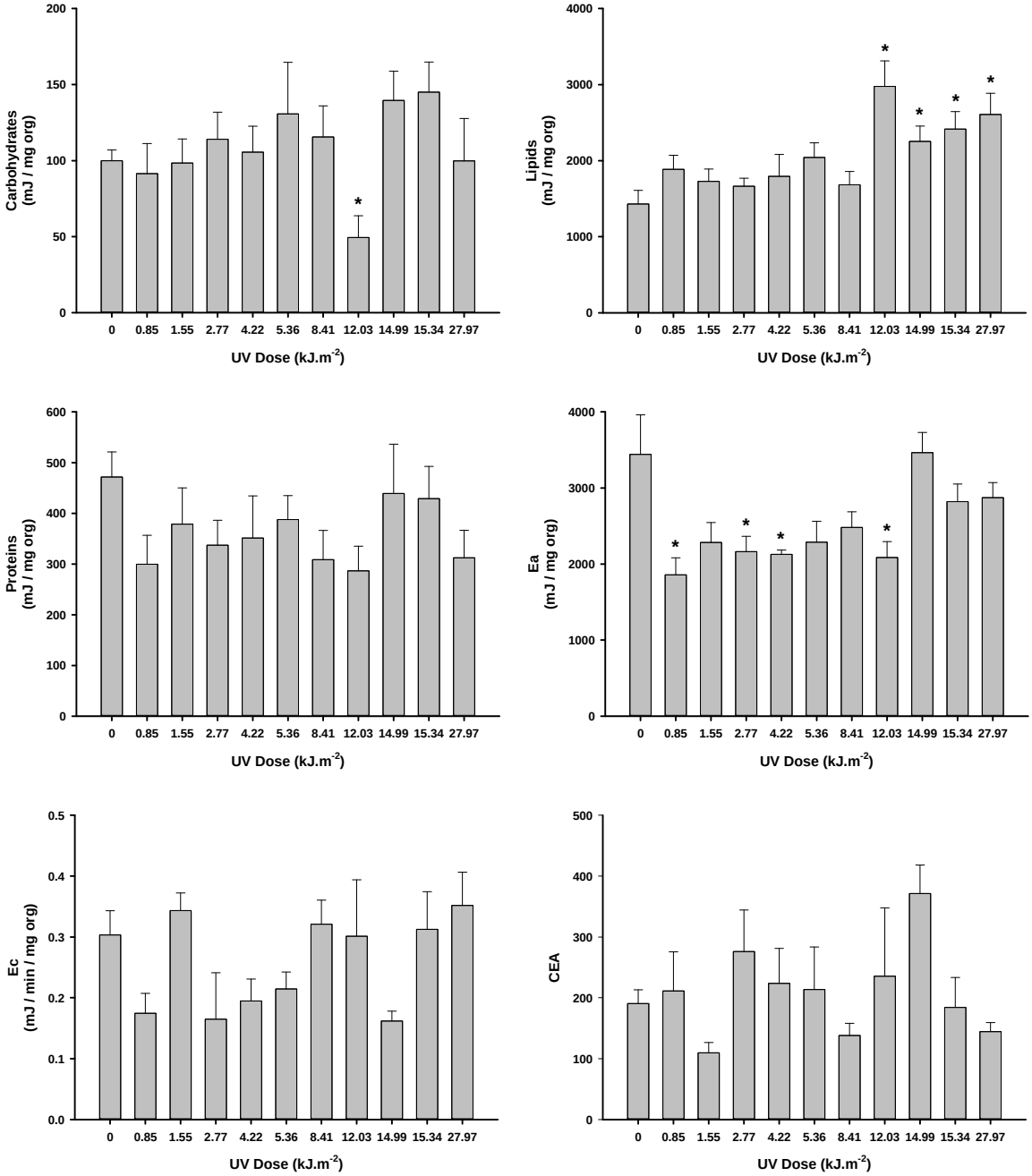

Fig. 2.6 Energy related parameters: lipids, carbohydrates and proteins content, energy available (Ea), energy consumption (Ec) and cellular energy allocation (CEA) of *Porcellionides pruinosus* exposed to different ranges UV (dose corrected for DNA damage). *- denotes significant difference when compared to the control.

4. Discussion

4.1. Temperature induced effects

One of the most important factors that can highly affect terrestrial isopods is temperature by influencing their water balance regulation, but also by indirectly affect dehydration/desiccation (Warburg, 1965). These organisms can significantly increase their evapotranspiration rates as a mean to decrease their body temperature during exposures to elevated temperatures, but it can hardly be beneficial for prolonged exposure periods, like a 14 days exposure (Edney, 1951). In this way, the additional effects of a severe dehydration must be also considered here. Results obtained for temperatures lower than the control (lower than 20ºC) exhibited almost no significant variations, nor any clear patterns on the biomarkers evaluated, that could evidence a mode of action (MoA). These results are in accordance with similar tolerance responses observed in previous studies for the species *Porcellio scaber* (Schuler et al., 2011) and for *Porcellio laevis* (Folguera et al., 2009). On the other side, the increase of temperatures seems to induce oxidative stress mainly at 30ºC. This is evidenced by GST and CAT results, and in accordance with previous studies (e.g. Bagnyukova et al., 2007; Yang et al., 2010) where higher temperatures tend to negatively affect terrestrial isopods by inducing dehydration (Hadley and Warburg, 1986), which is associated with oxidative stress as discussed further on. It is noteworthy to state that the differences between pre-exposure and sampling times were observed almost exclusively for higher exposure temperatures and for oxidative stress biomarkers.

Another important feature to pinpoint for the exposure to higher temperatures is the increasing pattern of AChE (30ºC, 96 h of exposure). This type of response is still very unclear and unexpected, but not unique to this specific exposure. In other studies with isopods from the same species (*P. pruinosus*) the AChE activity was also induced by molluscicides and UV radiation (respectively Santos et al., 2010 and Morgado et al., 2013). A plausible explanation for this unexpected increase could be the production of the variant acetylcholinesterase readthrough (AChE-R), and not the common variant acetylcholinesterase synaptic (AChE-S), which is generally considered the main target of neurotoxicity compounds (Grisaru et al., 1999; Ohno et al., 2000).

The effects of temperature in energy reserves has been widely studied (e.g. Sroda and Cossu-Leguille, 2011; Verslycke and Janssen, 2002). In our study, no significant differences were observed in protein, lipids or carbohydrates contents when different temperature exposures were compared. Proteins and carbohydrates presented similar values as the ones reported by Ferreira et al. (2010) whereas lipids showed almost twice the values reported, which can be the main reason for the results obtained for Ea. As for Ec, although a significant decrease is observed for all temperatures and sampling times when compared to the pre-exposure, significant differences to control were registered for 25ºC and 30ºC. This seems to be in line with the hypothesis of a down-regulation of cellular metabolism during stress (Hand and Hardewig, 1996). According to França et al. (2007), the production of free radicals is closely related to the respiration rates in such a way that a considerable gain in survival can be achieved just by reducing the metabolism when exposed to any dehydrating agent. This would also match the oxidative stress results for CAT and GST, since higher effects were observed for temperatures higher than 20ºC. In a previous work, Edney (1964) reported a temperature-dependent increase in standard metabolic rate for the terrestrial isopods *Porcellio laevis* and *Armadillidium vulgare* that seems to counteract our previous hypothesis. Nevertheless, Carefoot (1993) also stated that, despite an increase in isopods' oxygen uptake may occur when experiencing short-periods of low humidity, a significant reduction is expected when under severe dehydrating conditions. This response from the organisms seems to be positively compensated since the organisms show a better CEA index than the control, suggesting that even though organisms use the diminution of the Ec rates as strategy, metabolic costs to handle temperature are still present.

The combination of the results obtained for the biomarkers and energy costs across the full range of temperatures seemed to be complementary. Temperatures higher than 20ºC showed a higher impact in biomarkers activity, indicating that oxidative stress was being originated by this stressor, and the energy related parameters showed also deviations and behavioural strategies to handle oxidative stress and to return to a homeostatic status. In fact, studies regarding the behaviour of isopods such as Shachak (1980) or Dailey et al. (2009) can potentially bring new insights to the responses observed in this study.

4.2. Soil moisture exposure

The study on the effects of low and high percentages of moisture in soils (as WHC) represents an important issue to be considered when evaluating the responses of terrestrial isopods. Unexpectedly, biomarkers measurements showed no significant responses that could indicate any oxidative stress (GST or GPx), physiological damage (LPO) or neurotoxicity (AChE). An exception is the pattern of CAT with a decrease with time of exposure, which is more marked at 20, 40 and 60% WHC and after 14 days of exposure. This is partly surprising since dehydration is known to induce oxidative stress (França et al., 2007) and no significantly differences were observed in those biomarkers.

Contrary to the biomarkers, the analysis of energy related parameters seems to indicate significant efforts from organisms to handle this stressor, particularly in the lowest and highest percentages of moisture (20% and 80% WHC). In the case of 20% WHC a significant decrease is observed for lipids, Ea, Ec and CEA, whereas the 80% WHC shows a significant decrease in Ec that will lead to an increase in the CEA index. Although not so clear for the 80% WHC exposure, this impact becomes more evident if taken in consideration the high mortality rate, suggesting other mechanisms, not considered in this study. Like most mesic/xeric isopod species, *P. pruinosus* has a hydrophilic ventral cuticle that makes it unable to avoid water absorption in overly humid environments (Sutton, 1980). This can lead to serious problems with their osmoregulation making it prone to water overload. In a study performed by Zimmer (2004), alterations in soil moisture due to precipitation would alter breeding strategies resulting in "*reduced faunal contributions to decomposition processes that, in turn will lead to slowed-down microbial degradation of detritus and nutrient cycling*". The energy related parameters measured in this study, also suggest the same type of response, as organisms appear to spend more energy in soils with low moisture contents.

High mortality was observed for the higher soil moisture contents (80% WHC), which can be justified by the fact that *P. pruinosus* is a species inhabiting environments with higher temperatures and low soil moisture. For moistures higher than 60% WHC, these organisms can be considered unadapted and there is a very close gap between the optimal percentage of moisture and mortality. As for the percentages lower than 60% WHC, organisms are more

adapted and the turnover point between the optimal percentage of moisture and mortality is wider, making evident this species' adaptation to more arid environments.

The lack of a similar approach using biomarkers, energy related parameters to assess the effects of moisture on terrestrial organisms, makes the comparison of results very difficult. The majority of studies that involve moisture variation is related to environmental adaptations (e.g. Coenen-Staβ, 1981; Shachak, 1980; Zimmer, 2004), water regulation (e.g. Bursell, 1955; Riegel, 1959), seasonal variation (e.g. Edney, 1968) or focusing on atmospheric humidity (e.g. Allee, 1926; Gunn, 1937; Wolsky, 1933). On the other hand, despite the scarcity of similar studies, behavioural studies on the effects of moisture on terrestrial isopods are abundant and can bring some insights to the observed results. One example is the desert isopod *Hemilepistus reaumuri* that uses two behavioural strategies to deal with the low and unpredictable water resources and maintain the population, which include a borrow strategy and/or an increase in their reproduction rate to overcome higher mortality rates (Shachak, 1980). Therefore, studies based on endpoint like mortality, avoidance, feeding rates and locomotion can provide new insight on isopods responses to changes in soil moisture.

4.3. UV-R exposure

The effects of UV-R in terrestrial isopods have not yet been thoroughly studied, mainly due to the fact that these organisms have an inherent burrowing behaviour or look for shelter under rocks or leaf litter. In addition previous studies are mainly based on aquatic organisms (e.g. Ribeiro et al., 2011), making difficult the analysis and discussion of these results. Although terrestrial isopods are claimed to be not affect by UV radiation exposure, the results obtained in this work show that this is not entirely true. This is also supported by the study of Morgado et al. (2013) that evaluated the effects of UV-R in juveniles and adults isopods of *P. pruinosus*, showing a negative impact in biomarkers and energy reserves. Another study with the collembolan species *Folsomia candida* exposed to UV-R showed an increase in reproduction, but when combined with the pesticide carbaryl an independent action pattern was observed, previewing an impact in this species population (Cardoso et al., 2014).

For AChE activity, an inhibition pattern is followed by an induction reaching up to two times the control activity, showing an "inverted bell shape" enzymatic response. Although the inhibition of this enzyme activity has already been observed in previous studies (e.g. Souza et al., 2010), the induction observed for the last two doses were not expected. In fact the induction of AChE is not a commonly observed although been reported previously for the fresh water snail *Lymnaea stagnalis* (Mills et al., 1992; Mills et al., 1990), in various cell types (Zhang et al., 2002) and also for the same species used in our study the isopod *P. pruinosus* (Morgado et al., 2013; Santos et al., 2010). Possible explanations for this induction have already been presented in the previous section.

The mechanism of AChE inhibition due to UV-R, although not clearly understood, is proposed by Bishop et al. (1980) to be a result from the protein conformation alteration leading to its inactivation. Nevertheless, other types of physiological responses may also influence this inhibition, such as those related with the enzyme production, where UV-R may directly or indirectly (by ROS stress) act on the production pathway. The obtained results support the idea that UV-R affects neurotransmission, which will negatively impact for example on predatory avoidance behaviour or the energy budget of the organism.

As reported previously, UV-R is known to induce reactive oxygen species (ROS), being an effective prooxidant agent (Sinha and Hader, 2002). The pattern observed for GST activity is also a typical "bell-shape" enzymatic activity. This activity pattern complements the effort with CAT activity to handle UV-R stress, which was also observed by Morgado et al. (2013), but different from other studies such as the one from Vargas et al. (2010). The LPO rate also comes in accordance with both enzyme activities, where the only significant difference was found for the highest UV dose, with a significant decrease in LPO. This result suggests that, in this particular case, UV-R would have a compensatory effect in the organism, contradictory to all the stress that is being observed for all the other biomarkers and doses of exposure. In fact this result is not observed for other organisms (Novales Flamarique et al., 2000). The explanation for this reduction in LPO rate may lie on the non-enzymatic antioxidant protection compounds, which may mask our results preventing a clear observation of the real impact of UV-R, or may be a result of other mechanisms within the antioxidant protection system, where peroxidised lipids are being removed from the membrane (Davies, 2000).

The energy related parameters also pose some interesting results, where carbohydrates and lipids showed an increase pattern with the increase of exposure time, with carbohydrates showing a significant decrease at the same exposure time where the highest content of lipids was observed (4 h of exposure). Besides this specific case, no depletion on any of the three types of energy reserves was observed during exposure, which indicates that although some effort was being allocated to handle UV-R stress, it was not being noticeable on these organisms' energy reserves content. In this way, the analysis of the total available energy poses here an important data to better understand the real effects of UV-R on the energy reserves content. In fact, most of the UV-R doses are significantly lowering the energy budget available for the organisms and possibly an effort was being performed in order to deal with UV-R stress. The short exposure period with a maximum of 8 h and the lack of food in the exposures boxes, can hardly be taken as a possible explanation for these results since the non-availability of food is not expected to play any major role in the decrease of the energy reserves content in such a short period of time. The increase in energy consumption is also not a likely explanation as no significant differences were observed for all treatments. In the same way, the CEA index did not show any significant differences in all treatments. The negative effects of the total available energy decrease due to the effort to deal with the UV-R stress may result in the impairment of other energy related processes such as feeding, growth or even reproduction. Such influence in these organisms' life traits may be even amplified at population or ecosystem levels.

5. Conclusions

The present study presents the effects of temperature, soil moisture and UV-R in terrestrial isopods. Increases in temperature are expected to pose some concerns regarding life traits and more specific strategies to manage isopods' energy budget. In fact, these case scenarios will require from the organisms a higher consumption of energy in order to deal with oxidative stress and a rearrange their defence strategies, by lowering their metabolism, which will possibly be reflected in other traits, like locomotion. This species of isopods showed to easily acclimate to lower moisture scenarios. This work, although not presenting any significant changes on the organisms' metabolism, brings two important noteworthy points: 1) the species *P. pruinosus* is more adapted to low contents of water in soils; 2) in cases of

flood scenarios turnover point between optimal conditions and mortality can be considered narrow, and may lead to the local extinction of populations in specific micro-habitats. This study showed that UV-R should not be discarded as a possible abiotic stressor for isopods as it can negatively influence their life traits or be a cause of mortality. Although the majority of significant impacts were observed for the higher doses, one cannot discard that these conditions may occur in the next years, and scenarios of extreme summers are a reality nowadays.

In a very brief conclusion of this study, two points should be noteworthy: 1) the effects of abiotic factors should also be included and take into consideration by policymakers; 2) the inclusion of abiotic effects in ecotoxicological tests should be included in the analysis of any stressor to improve chemical risk assessment.

Acknowledgment

This study was supported by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia within the research project CLIMAFUN- CLImate Changes and Potential Impact on Soil FUNctional Ecology (FCOMP-01-0124-FEDER-008656) and through a PhD grant (SFRH/BD/65739/2009) awarded to Nuno G. C. Ferreira by FCT. The authors would like to thank for the technical support and advice given by Dr. Abel Ferreira.

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CHAPTER III

Biomarkers and energy reserves in the isopod Porcellionides pruinosus: The effects of long-term exposure to dimethoate.

Biomarkers and energy reserves in the isopod *Porcellionides pruinosus*: The effects of long-term exposure to dimethoate

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Published by Science Of The Total Environment 502, 91-102. doi:10.1016/j.scitotenv.2014.08.062

Abstract:

Terrestrial isopods from the species *Porcellionides pruinosus* were exposed to the recommended field dose application (0.4 mg/kg soil) and a sublethal concentration (10 mg/kg soil) of dimethoate at two temperatures that can be generally found in several countries (20ºC and 25ºC) and are commonly used as reference temperatures. The organisms were exposed for 28 days and sampled at the following time points: 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days; organisms were then changed to clean soil for a recovery period of 14 days during which organisms were sampled on day 35 and 42. For each sampling time, the enzyme activities of acetylcholinesterase (AChE), glutathione-*S*-transferases (GST), catalase (CAT), lactate dehydrogenase (LDH) were determined as well as the following: total lipid, carbohydrate and protein content; energy available (Ea); energy consumption (Ec); cellular energy allocation (CEA) and lipid peroxidation rate (LPO). The integrated biomarker response (IBR) was calculated for each sampling time and for each of the above parameters. Mortality was also recorded during the study.

The results obtained showed that dimethoate causes toxicity by several mechanisms. This study found evidence for the inhibition of the acetylcholinesterase enzyme, which has been previously reported, and also evidence of oxidative stress, which altered the levels of GST, CAT or LPO. In addition, the study showed that the two concentrations used of dimethoate caused the activation of different general detoxification mechanisms, and also that the same concentration at different temperatures induced different toxicity responses.

***Keywords*:** integrated biomarker response, oxidative stress, neurotoxicity, combined effects, temperature changes.

1. Introduction

The potential impact of a stressor in ecosystems requires the observation of effects at different levels of biological organization, starting at the molecular level and ending at the population/community level (Moore et al., 2004). At a molecular level, several biomarkers have been used as efficient tools due to their sensitivity, quickness and accurate relationship between toxicant exposure and respective biological response (Morgan et al., 1999). However, results from molecular approaches may have limited information if they are not related to higher and more complex biological levels of organization. Indeed, the effects at lower organizational levels may not necessarily be observed or be meaningful at superior biological levels whenever they consist on the acquisition of a new homeostasis status (Morgan et al., 1999), or they just do not represent any major life-changing effects. Nevertheless, they can provide crucial information on stressors' modes of action, which improve the knowledge on their related effects. Depletion of energy reserves and energy metabolic costs can be used as another parameter to evaluate chemical exposure effects. Due to the stress induced by xenobiotics, metabolic changes can induce the depletion of energy reserves especially under long time exposures, negatively affecting individuals' growth or reproduction, and finally impairing population dynamics and structures (de Coen and Janssen, 2003).

In the soil compartment, organisms play an important role on decomposition and fragmentation processes, and their exposure to xenobiotics may change overall soil functions, causing a decrease in soil quality and soil services (MEA, 2005). There is a wide range of xenobiotics that can appear in the terrestrial compartment. Organophosphorous compounds (OP) are one of the most extensively used pesticides in agriculture practices. One of the most commonly applied is dimethoate, which successfully combines a selective toxicity to insects through a systemic action. By acting on the enzyme AChE, this pesticide inhibits the degradation of acetylcholine thereby producing extensive cholinergic stimulation and neurotoxicity (de Coen and Janssen, 2003). When looking at xenobiotics'

exposures, the overall conditions of exposure can also bring additional stress or induce changes in physiological responses of organisms when they are dealing with chemical exposure.

Terrestrial isopods are macrodecomposers that play an important role in the decomposition processes, vegetal litter fragmentation and re-cycling process of nutrients (Ferreira et al., 2010; Loureiro et al., 2006; Zimmer, 2002; Zimmer et al., 2003). The terrestrial isopod species Porcellionides pruinosus has been described as a good test-organism to evaluate soil contamination or other environmental changes in their habitat (Jansch et al., 2005; Loureiro et al., 2009; Loureiro et al., 2005; Takeda, 1980; Vink et al., 1995). Several individual parameters have been chosen as indicators of isopod health status but also as parameters tightly related to their function in soils. Feeding activities, including excretion rates, reproduction, growth and behaviour are amongst the most used parameters in isopods ecotoxicological tests. Along with these, the use of neurotoxicological (acetylcholinesterase), detoxification (glutathione S-transferases), oxidative stress (catalase, glutathione peroxidase, and lipid peroxidation), energy related (lactate dehydrogenase) biomarkers' basal activities and energy reserves (total lipid, carbohydrate and protein content) can be used as good evaluation tools that will provide useful information and a connection between these two ecological levels. In previous studies, these biomarkers have been used to determine the basal levels on organisms from well-established lab cultures (Ferreira et al., 2010) and they have also shown to respond to the short term exposure of pesticides (e.g. Jemec et al., 2009; Jemec et al., 2012).

Time of exposure is one of the key factors to improve ecological relevance. In isopod bioassays, tests are usually carried out for 48h, when considering the avoidance behaviour test (Loureiro et al., 2002), 14 or 28 days when regarding feeding inhibition tests (Loureiro et al., 2006), 14 days for survival (Calhôa et al., 2012; Santos et al., 2010), 21 days for bioaccumulation tests (Sousa et al., 2000), or more than one month when reproduction is being evaluated (Calhôa et al., 2012). Long term exposure tests are advisable when a more comprehensive approach is required in order to integrate chemical fate and changes in bioavailability with time, but also to consider the ability of organisms to recover when exposure ends.

Therefore, the main goal of this study was to evaluate and understand the long-term effects of dimethoate using several enzymatic biomarkers and energy reserves in the terrestrial isopod Porcellionides pruinosus. Organisms were exposed to two dimethoate concentrations (a recommended field dose application and a concentration below EC50 level) and two different exposure temperatures (20ºC and 25ºC) during a 28 day exposure period followed by a 14 day recovery period. The results were then combined using the integrated biomarker response index (IBR).

The mode of action (MoA) analysis of dimethoate to the terrestrial isopod *P. pruinosus* was determined in several ways: 1) the toxicity and inherent effects of two dimethoate concentrations; 2) the toxicity effect of dimethoate combined with temperature; 3) response patterns at the different times of exposure and 4) differences between the exposure and recovery period. Within this approach, dimethoate degradation in soils was also integrated in the results in the presence or absence of terrestrial isopods.

2. Materials and methods

2.1. Test organism and culture procedure

The organisms used in this study belong to the species *Porcellionides pruinosus* Brandt (1833), and were previously collected from a horse manure heap and maintained for several generations in laboratory cultures. In culture, isopods were fed *ad libitum* with alder leaves (*Alnus glutinosa*) and maintained at two different temperatures (20 ± 1°C and 25 ± 1°C), with a 16:8h (light:dark) photoperiod. Twice a week cultures were sprayed with water and food provided. Only adult organisms (15-25 mg wet weight) were used in the experiments and no distinction between sexes was made, although pregnant females were excluded.

2.2. Soil spiking

LUFA 2.2 soil (Speyer, Germany) was spiked with two different concentrations of dimethoate (0.4 and 10 mg dimethoate/kg soil), with a final moisture content equivalent to 50% of the soil water holding capacity. The concentration of 0.4 mg dimethoate/kg soil represents the recommended field dose for dimethoate application (TitanAG, 2010) (Santos et al., 2011)(Santos et al., 2011)and the 10 mg dimethoate/kg soil was used based on the study by Fischer et al. (1997) that found in LUFA 2.2 soil for the isopod *Porcellio scaber*, a EC50 value of 17.5 mg dimethoate/kg soil for growth; 16.8 mg dimethoate/kg soil for mancae/surviving females and 15.4 mg dimethoate/kg soil for pregnant/surviving females. Therefore, the concentration of 10 mg dimethoate/kg soil was chosen as a sublethal value.

2.3. Experimental procedure

Toxicity tests were performed in plastic boxes (26 length x 18 width x 7.5 height cm), containing approx. 2cm height of natural LUFA 2.2 soil (Speyer, Germany) and 40 isopods (per box). Organisms with abnormalities, moulting characteristics or pregnant females were excluded from trial. Although food was provided *ad libitum* in the form of alder leaf disks (Ø 10 mm), it was made available considering also that a soil coverage by leaves could influence isopods' exposure to the spiked soil. Therefore, food was added in small quantities but continuously reintroduced throughout the test period. Organisms were exposed to 0.4 and 10 mg dimethoate/kg soil in a 16:8h (light:dark) photoperiod, at two different temperatures: 20ºC and 25ºC. Both temperatures are relevant temperatures for Mediterranean countries, and can be found elsewhere during a year time and are widely used in ecotoxicological assays with several species of terrestrial isopods (Calhôa et al., 2012; Dailey et al., 2009; Loureiro et al., 2006; Morgado et al., 2013; Ribeiro et al., 1999; Santos et al., 2011).

A total of five replicates were performed for each concentration and temperature. Four organisms from each box/replicate were collected at the following time points: 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days (exposure period) and 35 days, 42 days (recovery

period). In the results section, the 35 and 42 days of test duration will be denominated as 7 and 14 days of post-exposure.

The enzymatic biomarkers glutathione *S*-transferases (GST), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LPO) were measured using a pool of two full-body organisms per replicate. Another organism was divided into head and body to analyse acetylcholinesterase (AChE) and lactate dehydrogenase (LDH) activity, respectively, each part corresponding also to a replicate. All chemicals used in these experiments were obtained from Sigma-Aldrich Europe, except the Bradford reagent, which was purchased from Bio-Rad (Germany), and hydrogen peroxide from Fluka.

For the energy reserves (total lipid, carbohydrate and protein content) and electron transport system, only one organism was used as a replicate.

At each sampling time, the number of dead organisms were recorded and removed from the test boxes.

2.4. Measured parameters and IBR

The protocol used to process samples was previously described by Ferreira et al. (2010) and is extensively described in the supplementary data. The lipid peroxidation (LPO) assay was adapted from the methods described by Bird and Draper (1984) and Ohkawa *et al*. (1979) to a microplate format. The glutathione *S*-transferases (GST) and glutathione peroxidase (GPx) activities were determined as described by Habig *et al*. (1974) and Mohandas *et al*. (1984), respectively. Catalase (CAT) activity was determined based on the method described by Clairborne (1985) and adapted to a microplate format. Lactate dehydrogenase (LDH) activities were measured using the method described by Vassault (1983), adapted to microplate format by Diamantino et al. (2001) and the acetylcholinesterase (AChE) activity according to the Ellman method (Ellman et al., 1961), adapted to microplate format described by Guilhermino et al., (1996). For all biomarkers, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's

Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as a standard.

Considering energy parameters, to determine total protein, carbohydrate and lipid contents, oxygen consumption rate in the electron transport system (energy consumption - Ec), energy available (Ea) and cellular energy allocation (CEA) protocols were adapted from de Coen and Janssen (1997). The Ea, Ec and CEA value were calculated as described by Verslycke et al. (2004):

Ea (available energy) = carbohydrates + lipids + proteins (mJ / mg org.)
Ec (energy consumption) = ETS activity (mJ / mg org. / h)
CEA (cellular energy allocation) = Ea/Ec (/h)

To integrate results from the different biomarkers and understand global/general responses, the integrated biomarker response (IBR) was calculated according to Beliaeff and Burgeot (2002); details can also be found in the supplementary data.

2.5. Chemical analysis

Determination of total dimethoate concentration per kilogram of soil was performed by the Marchwood Scientific Services Ltd. The lower detection limit for dimethoate was 0.4 µg/kg soil. The method used to analyse soil spiked with dimethoate involved air drying and grinding the samples. Then 0.5 gram of sample was used for extraction with acidified acetonitrile. The sample was then filtered and the filtrate used for analysis by Liquid Chromatography-Tandem Mass Spectrometry following a pre-treatment buffering stage. The instrument used for the analysis was an Agilent 6410 Triple quad LCMS-MS. Standards were prepared in solvents at 7 levels with recoveries in the range of 80 - 120%. The water sample analysis passed for all the samples except the drying and grinding stages.

Soil samples analysed consisted of a pool of 5 soil replicates for each concentration used to expose isopods. Similarly, soil spiked with the same concentrations of dimethoate but with

no isopods was also sampled. Samples were taken at the beginning of the exposure, and at days 7, 14, 21 and 28.

2.6. Data analysis

A one-way analysis of variance (ANOVA) or a Student test (*t test*) was performed to compare differences between treatments at each sampling time and Dunnett's comparison test was carried out to discriminate statistically different treatments from the control (SPSS 1999). When possible, data transformation was used to achieve normality. When data did not show a normal distribution or homoscedasticity, the non-parametric test Kruskal-Wallis One Way Analysis of Variance on Ranks was used.

Data values that were higher or lower than the mean value, plus or minus two times the standard deviation, were considered outliers, and withdrawn from analysis (Rousseeuw et al. 1993). Whenever there was enough data (n>3, due to high mortality rates), a two-way analysis of variance (two-way ANOVA) was performed to check for interactions between time and concentration. The two-way ANOVA was performed separately for the exposure and the recovery period. The one-way ANOVA and two-way ANOVA with significance of $\alpha = 0.05$. Due to the mortality observed within each temperature, under 20ºC a two-way ANOVA could only be performed for the exposure period and for the 25ºC exposure for the recovery period.

Dimethoate decay on time was calculated using non-linear regression curves at the two different temperatures, with and without the presence of isopods, and calculated as an exponential single decay curve with 2 parameters ($C_t = C_0 e^{-K_0 t}$), where C_t is the dimethoate concentration in soil (mg/kg soil), C_0 is the initial concentration of dimethoate in soil, K_0 the decay rate of dimethoate in soil (/day), and t the time (days).

3. Results

Organisms exposed to dimethoate under the two different temperature regimes (20ºC and 25ºC) showed different mortality patterns. In Fig. 3.1 it is presented the number of dead organisms and the cumulative number at a given sampling time. In the 20ºC exposure regime, due to the mortality observed at the highest concentration, on the 14th day of recovery it was not possible to analyse the energy related parameters (proteins, lipids, carbohydrates, Ec, Ea and CEA). Similarly in the 25ºC exposure regime, the high mortality observed for the highest concentration did not allow the analysis of the energy related parameters (proteins, lipids, carbohydrates, Ec, Ea and CEA) for the 14th day of exposure and all parameters forward.

The decay rate for the lower and higher concentrations of dimethoate in soil at 20ºC with isopods present was respectively 0.32/d ($y = 0.4e^{-0.3216x}$; $r^2 = 0.9994$) and 0.13/d ($y = 9.9e^{-0.1277x}$). For the exposure at 20ºC where no isopods were present, no data is available due to analytical constraints.

The decay rates of dimethoate in soil at 25ºC are presented in Fig. 3.2. At the lower concentration, the decay rates for soil with and without isopods were respectively 0.37/day ($y = 0.4e^{-0.3665x}$ $r^2 = 0.9987$) and 0.33/day ($y = 0.4e^{-0.3259x}$; $r^2 = 1.0000$). For the higher concentration, those rates were 0.29/day ($y = 10e^{-0.2921x}$; $r^2 = 1.0000$) and 0.13/day ($y = 9.9e^{-0.1315x}$; $r^2 = 0.9903$), for soil with and without isopods.

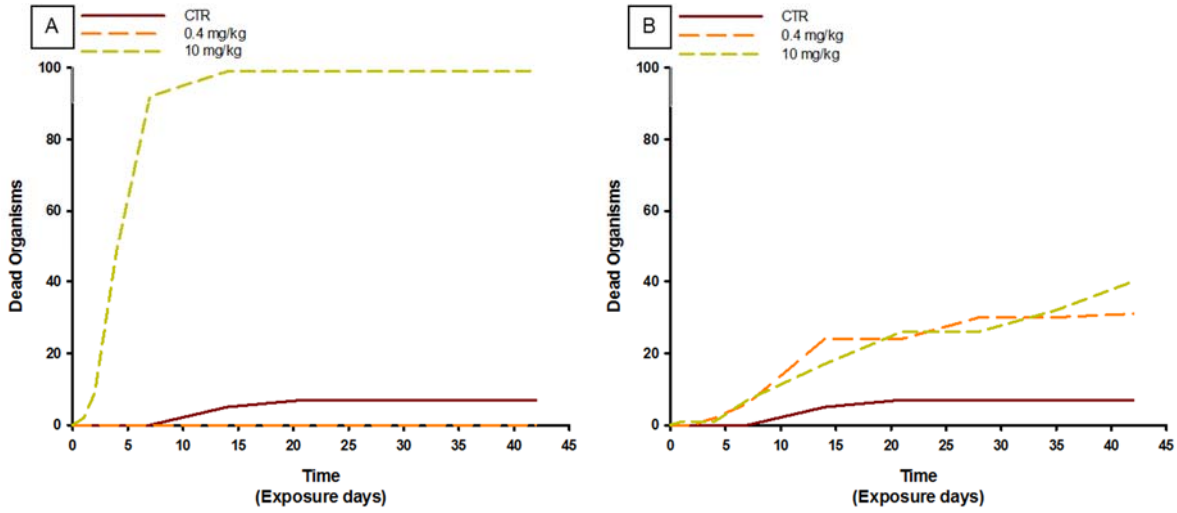

Fig. 3.1 Cumulative total number of dead organism from the species *Porcellionides pruinosus* during the exposure and recovery period in the control and exposed to 0.4 mg dimethoate/kg soil and 10mg dimethoate/kg soil. A total of 200 organisms were exposed per treatment. A - organisms exposed to 20ºC, B - organisms exposed to 25ºC

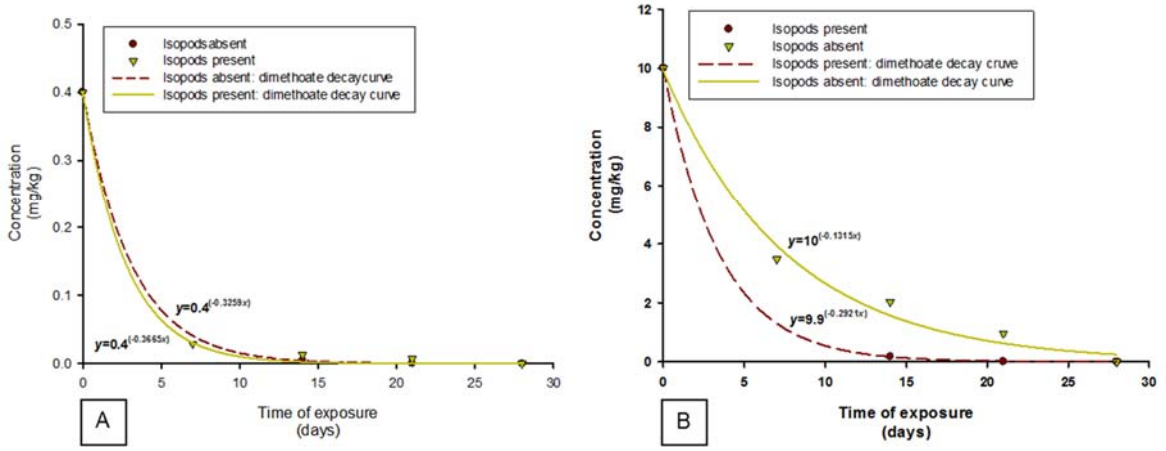

Fig. 3.2 Dimethoate decay curve for soil spiked with 0.4 mg dimethoate/kg soil (A) and 10 mg dimethoate/kg soil (B) at 25ºC exposure in the presence and absence of terrestrial isopods.

3.1. Biomarkers activity and energy reserve content

The activity of the biomarkers and energy reserves of organisms exposed at 20ºC and 25ºC during the exposure and the recovery period is presented in Fig 1SD and 2SD (supplementary data) and in Table 3.1. Details relative to the significant differences found between treatments and control are presented as supplementary data (Table 2SD).

The main target enzyme of the pesticide dimethoate (AChE) showed significant differences mainly at the higher concentration (10 mg dimethoate/kg soil) for both temperatures. The lower exposure concentration only showed significant differences at 20ºC after 48h of exposure. Regarding the oxidative stress related biomarkers (LPO, GST, CAT and GPx), significant differences were mainly observed at 25ºC exposure and after 48h/96h of exposure or during the recovery period (more evident at the higher exposure concentration). Finally the energy related enzyme LDH presented significant differences at 20ºC for both concentrations during the 7/14 days of exposure.

The significant differences observed at 25ºC on the energy related parameters were mainly found after 96h of exposure and mostly consisted on positive effects (except for the energy consumption – Ec), whereas for 20ºC negative effects were registered, in particular after 21 days of exposure (except for CEA after 48h and 96h of exposure).

At 20ºC, significant interactions between time of exposure and dimethoate concentrations were observed for the energy consumption (Ec), energy available and CEA in the exposure period (two-way ANOVA, ln transformation, $F_{14,75} = 2.967$; $p = 0.001$; two-way ANOVA, ln transformation, $F_{14,79} = 2.355$; $p = 0.009$ and two-way ANOVA, $F_{14,68} = 3.586$; $p < 0.001$, respectively) and for catalase in the recovery period (two-way ANOVA, $F_{4,24} = 5.180$; $p = 0.004$).

At 25ºC, significant interactions between time of exposure and dimethoate concentrations were only observed in the recovery period for LPO, GST, LDH and lipids content (respectively two-way ANOVA, $F_{2,20} = 5.727$; $p = 0.011$; two-way ANOVA, $F_{2,24} = 5.727$; $p = 0.009$; two-way ANOVA, $F_{2,22} = 9.343$; $p = 0.001$ and two-way ANOVA, $F_{2,21} = 3.959$; $p = 0.035$).

Table 3.1 Significant differences found for biomarkers and energy reserves between control organisms from the species *Porcellionides pruinosus* and those exposed to dimethoate at 20ºC and 25ºC. Data refers to a 28 day exposure period followed by a 14 day recovery period. Red boxes denote deleterious effects, green boxes denote positive effects and grey boxes represent parameters that could not be measure due to the lack of organisms. ↗ denotes significant increase, ↘ denotes significant decrease; One Way ANOVA, ANOVA on Ranks or Student's t-test, $p \leq 0.05$.

			AChE		GST		LPO		CAT		LDH		Lipids		Carbohydr.		Proteins		Ea		Ec		CEA	
			20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC
Exposure	24h	0.4 mg/kg									↘													↗
		10mg/kg	↘	↘							↘												↗	
	48h	0.4 mg/kg	↘			↗					↗											↗		
		10mg/kg	↘	↘		↗				↘	↗				↘									
	96h	0.4 mg/kg										↗				↗		↗		↗				
		10mg/kg	↘	↘			↘			↗	↗				↘	↗				↗		↗	↗	
	7d	0.4 mg/kg																				↗		
		10mg/kg	↘	↘				↗			↘	↗						↘						
	14d	0.4 mg/kg									↗													
		10mg/kg	↘	↘																				
	21d	0.4 mg/kg			↗	↘							↘	↗					↘				↘	↗
		10mg/kg	↘										↘						↘		↗		↘	
	28d	0.4 mg/kg												↘			↘							
		10mg/kg	↘																					
Post-Exposure	7d	0.4 mg/kg							↘		↗	↗												
		10mg/kg	↘														↘							
	14d	0.4 mg/kg				↘		↗																
		10mg/kg							↘															

3.2. Integrated biomarkers response (IBR)

The IBR starplot is presented in Fig. 3.3 and Fig. 3.4, and includes the scores of each measured parameter and each sampling time during the exposure and post-exposure period. Better or worse scores (respectively lower and higher values) obtained for each parameter are summarized in Table 1SD (supplementary data).

3.2.1. Exposure 20ºC: IBR and IBR/n analysis

The IBR analysis showed frequently worse scores (higher values) for the highest concentration (10 mg dimethoate/kg soil) than the other treatments (Fig. 3.5 and Fig. 3.6). When analysing the IBR according to the sampling time (Fig. 3.5), one can see that only at sampling times 24h and 28 days of exposure this situation did not occur. Moreover, apart from day 7 and day 28 of exposure, the lower exposure concentration (0.4 mg dimethoate/kg soil) showed always worse scores than the control. When changed to clean soil for recovery this effect disappears and the lower concentration always presented better scores than the control. The absence of an IBR for the highest concentration at day 14 of the recovery phase is due to the aforementioned lack of animals to measure the complete set of parameters used in the remaining sampling times (high mortality). Organisms in the control treatment always showed better scores except for the 14th day of recovery. A statistical analysis showed that only the highest concentration (10 mg dimethoate/kg soil) is significantly different from the control (one-way ANOVA, $F_{2,26} = 3.678$; $p = 0.039$).

When analysing the parameters individually, organisms in the control treatment always showed better scores, except for LPO and CEA. For the lower concentration the parameters that exhibit the highest toxicity when compared to the control were energy consumption (Ec, 11.3x higher), GST (9.6x higher) and protein content (4.7x higher). For the highest concentration of exposure (10 mg dimethoate/kg soil), higher toxicity was observed for the energy consumption (Ec – 21.1x higher), AChE (16.7x higher) and GST (13.4x higher).

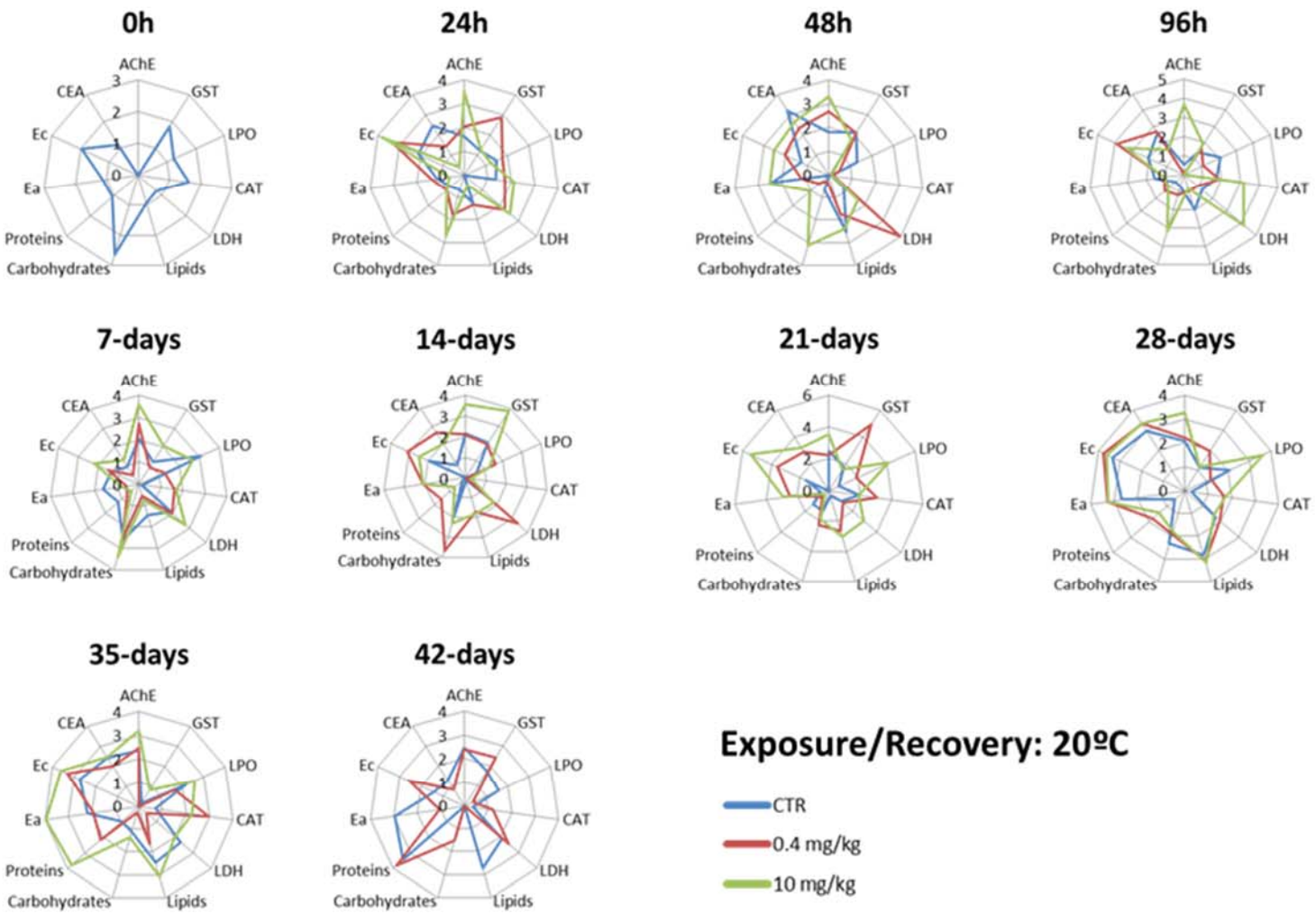

Fig. 3.3 Star plots for each sampling time of *Porcellionides pruinosus* exposed to 20ºC (exposure period: 0h, 24h, 48h, 96h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days, 42 days). AChE = acetylcholinesterase, GST = glutathione *S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

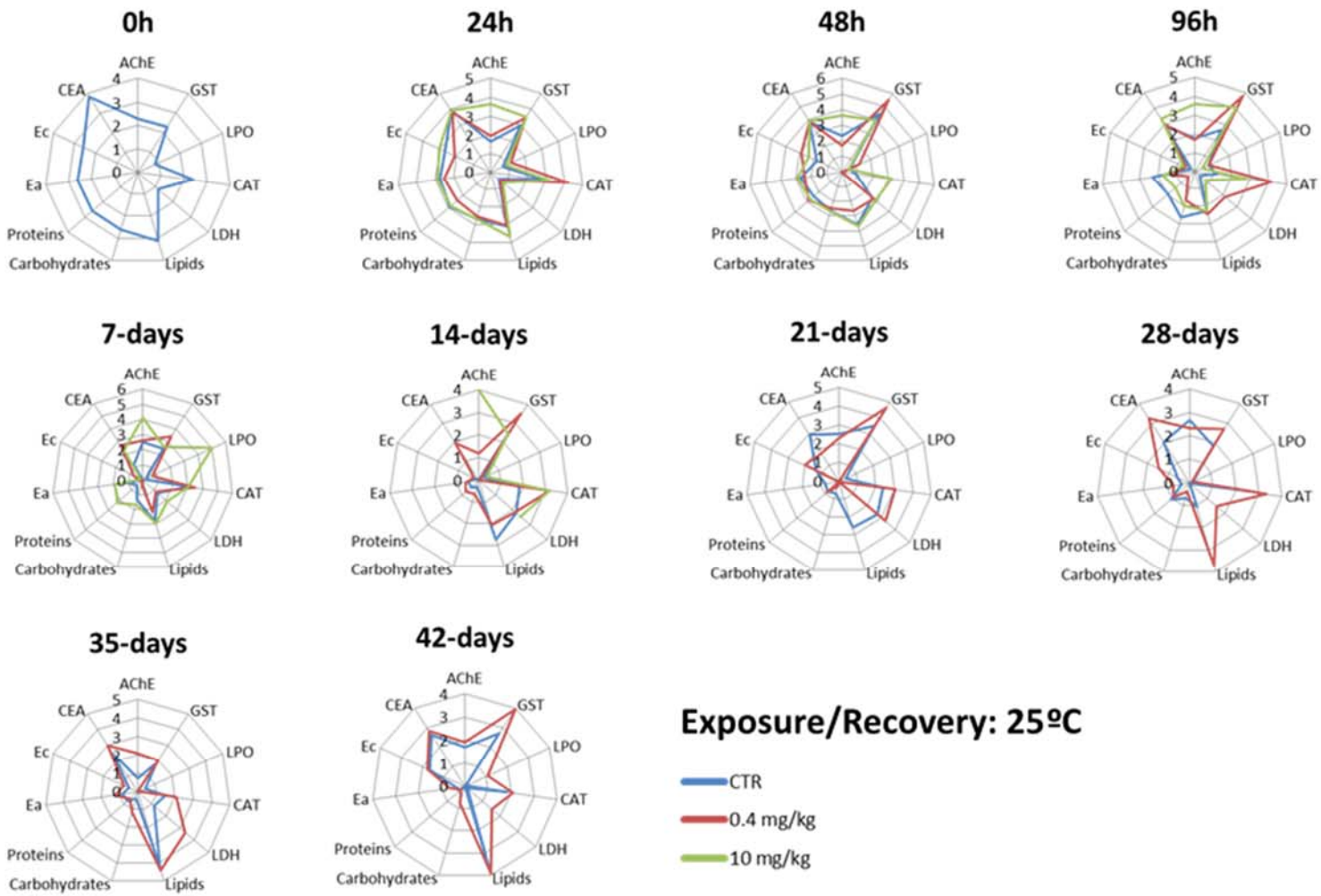

Fig. 3.4 Star plots for each sampling time of *Porcellionides pruinosus* exposed to 25ºC (exposure period: 0h, 24h, 48h, 96h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days, 42 days). AChE = acetylcholinesterase, GST = glutathione *S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

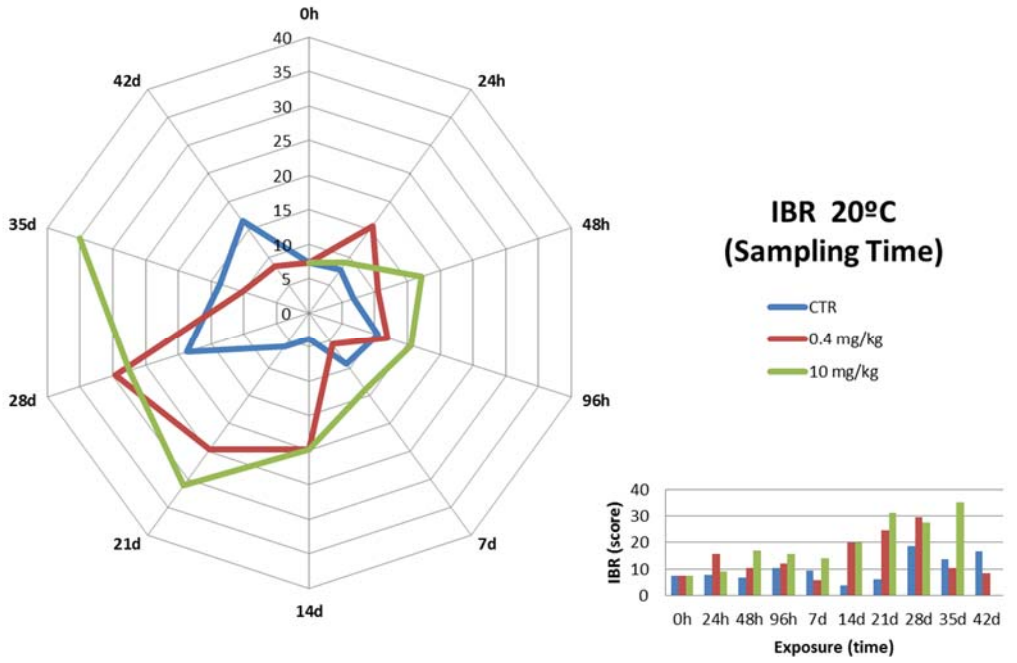

Fig. 3.5 Integrated Biomarker Response (IBR) represented by starplot and histogram of *Porcellionides pruinosus* in the control and exposed to dimethoate (0.4 and 10 mg/kg soil) at 20ºC. Exposure period: 0h, 24h, 48h, 96h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days, 42 days.

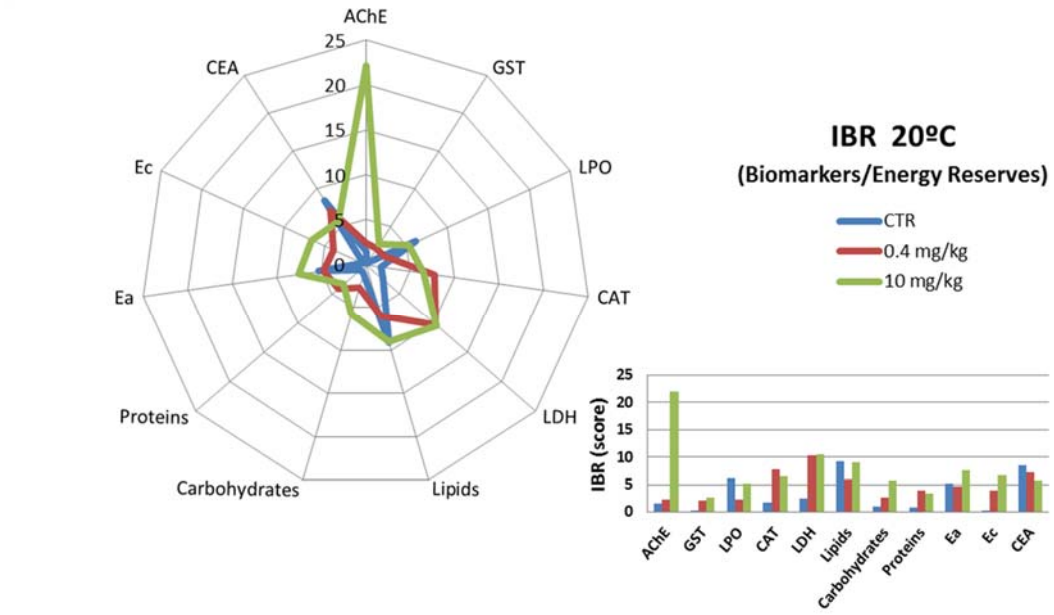

Fig. 3.6 Integrated Biomarker Response represented by starplot and histogram of *Porcellionides pruinosus* in control and exposed to dimethoate (0.4 and 10 mg/kg soil) at 20ºC during the exposure and recovery period. (AChE = acetylcholinesterase, GST = glutathione *S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation).

3.2.2. Exposure 25ºC: IBR and IBR/n

As in the 20ºC exposure, the IBR analysis showed always worse scores for the highest concentration used (10 mg dimethoate/kg soil) than the other treatments (Fig. 7 and 8). Also and except for the 24h and day 21 of exposure the lower exposure concentration (0.4 mg dimethoate/kg soil) showed always worse scores than the control, showing again a deterioration effect that increases with the exposure concentration (Figure 7). But, contrary to the 20ºC exposure, the change of organisms to clean soil for recovery did not show any positive effect. As it can be seen in Fig. 7, the IBR for the highest dimethoate concentration could not be assessed for sampling times from day 14 onwards of the exposure phase due to the increased mortality found at 25ºC. The statistical analysis also showed that only the highest concentration (10 mg dimethoate/kg soil) is significantly different from the control (Kruskal-Wallis, H=8.348; d.f. = 2; p=0.015).

For the lower concentration the parameters that exhibit the highest toxicity when compared to the control were CAT (11.0x higher), GST (7.4x higher) and LDH (6.7x higher) (Figure 8). For the highest concentration of exposure, the parameters that exhibits the highest toxicity were CAT (11.5x higher), AChE (10.8x higher) and LDH (7.6x higher).

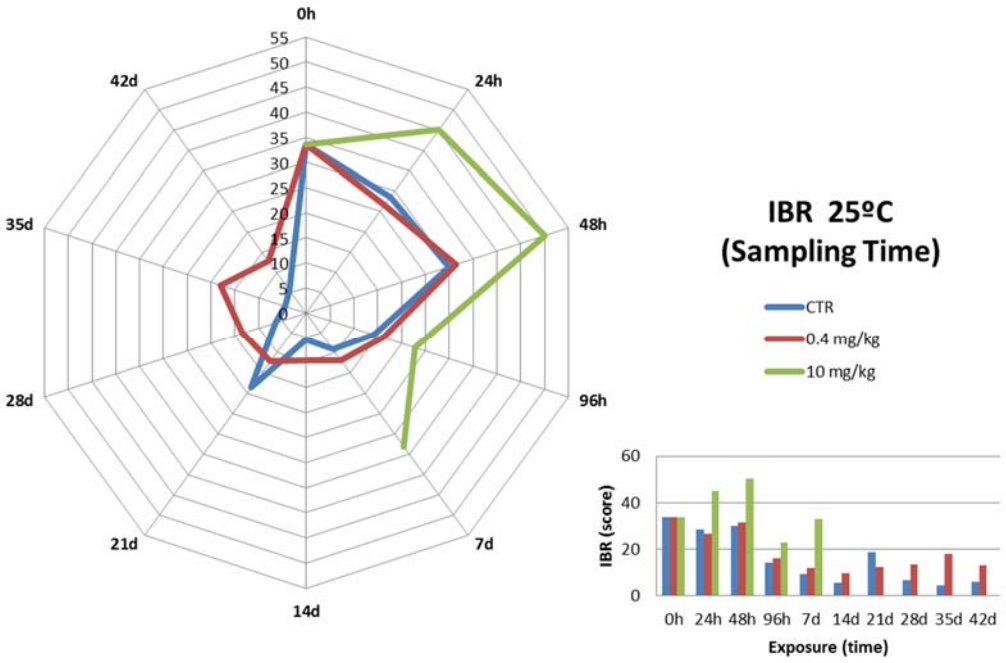

Fig. 3.7 Integrated Biomarker Response (IBR) represented by starplot and histogram of *Porcellionides pruinosus* in the control and exposed to dimethoate (0.4 and 10 mg/kg soil) at 25ºC. Exposure period: 0h, 24h, 48h, 96h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days, 42 days.

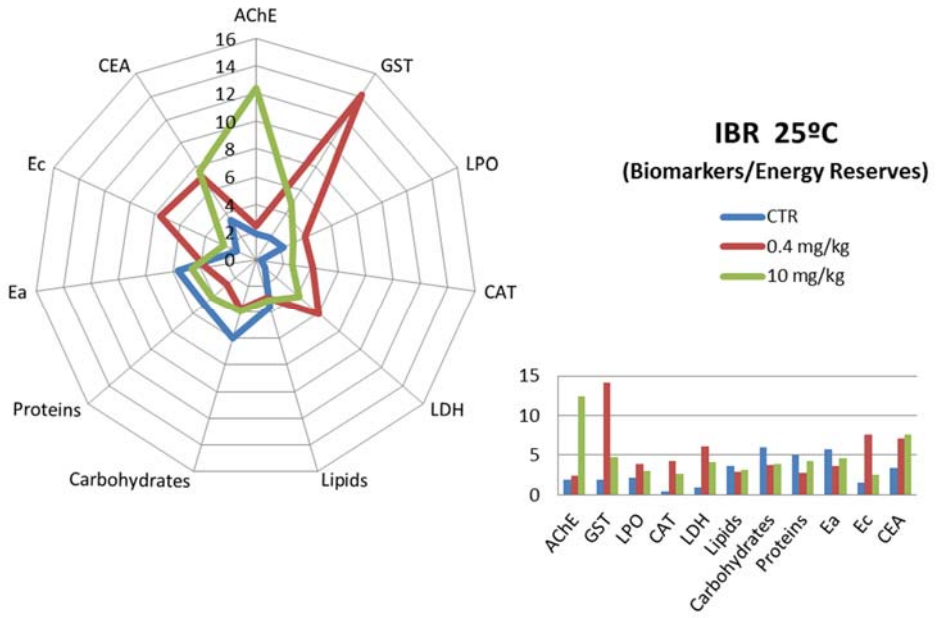

Fig. 3.8 Integrated Biomarker Response represented by starplot and histogram of *Porcellionides pruinosus* in control and exposed to dimethoate (0.4 and 10 mg/kg soil) at 25ºC during the exposure and recovery period. (AChE = acetylcholinesterase, GST = glutathione *S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation).

4. Discussion

The use of biomarkers to assess toxicity has already been used for a large number of species, scenarios and as a tool to determine which pathways are triggered by stressors, singly applied or as mixtures (e.g. Santos et al., 2011; Santos et al., 2010; Jemec et al., 2009; Jemec et al., 2012; Stanek et al., 2006). In these studies, the number of biomarkers used is normally high so the interpretation and integration of all their effects is difficult and further consequences for the organism are hard to determine. This analysis brings several problems in terms of interpretation, since nonexistence of significant differences to the control in one or several biomarkers may still be indicative of stress. In fact, an organism that does not show significant alterations from the control in all biomarkers from a specific pathway may be in a similar state to an organism that shows a significant difference in only one of the biomarkers. The use of IBR for a laboratory exposure and not as a tool to evaluate a specific field scenario has only been reported by Morgado et al., (2013). In the present study, the use of star-plots helped to identify patterns of toxicity that were not so clear when significant differences were analysed. And it proved to be a robust tool, as it could identify significant results between control and exposures in almost all cases, except one (CEA, 24h, 0.4 mg dimethoate/kg soil, 25ºC) representing 0.30% of total comparisons. An example of this was the AChE activity in organisms exposed to the lowest concentration at 20ºC. When looking only to significant differences between the treatment and control, organisms may be considered in "good conditions", but when analysing the IBR results an inherent toxicity pattern was observed.

In our results, one should highlight that organisms exposed to the highest concentration used (10 mg dimethoate/kg soil) showed a higher mortality at 25ºC. For the 20ºC exposure, mortality was not so high and therefore almost all sampling times could be fulfilled; in addition the observed mortality was very similar to the one found for the lower concentration (0.4 mg dimethoate/kg soil). These differences may be explained by faster degradation of dimethoate at 25ºC, which can lead to an increase of metabolites that can be more toxic than dimethoate (Lucier and Menzer, 1970; Martikainen, 1996).

Secondly another highlight from the present study was the strong inhibition of the enzyme acetylcholinesterase in the 10mg dimethoate/kg exposure. It is widely known that this

enzyme is the main target of dimethoate, and its inhibition was expected during the exposure. But an inhibition >90% was observed for the highest concentration for all sampling times at both temperatures. Such inhibition is generally considered to cause severe problems to the organisms and in some cases even death (Guimarães et al., 2007; Lucier and Menzer, 1970). The inhibition of the AChE activity was also observed in a recent work for two other species of earthworms, exposed to dimethoate, where concentrations corresponding to 25% of the field recommended dose inhibited up to 60% of this enzyme activity (Velki and Hackenberger). In addition, diazinon exposure to isopods via food led to approximately 50% and 90% inhibition, in adults and juveniles, respectively (Stanek et al., 2006). The negative effects of dimethoate in terrestrial isopods based on the locomotion impairment have been reported by Engenheiro et al. (2005), where an AChE inhibition of ~60% was correlated with a shorter path length travelled and more stops per path. Also some previous works using dimethoate and terrestrial isopods showed a higher AChE inhibition and high mortality (Santos et al., 2011; Santos et al., 2010).

This study also highlights the influence of isopods on the increase of dimethoate's degradation rate. This has been reported in a previous work by Loureiro et al. (2002) for the degradation of lindane in soil. The mechanism that underlies this faster rate of degradation of dimethoate in the presence of isopods may be related to their role in ecosystems. Their feeding on decaying vegetal matter and grazing on fungi leads to the release of faecal pellets that are enriched with bacteria present in their gut. This will increase the soil microbiome activity leading in a final step to a possible faster degradation of these organic compounds (Loureiro et al., 2002; Zimmer et al., 1999).

Regarding biomarkers, the enzyme GST, which is involved in the detoxification process, did not show any significant differences during the exposure and recovery period at 20ºC and at the lowest dimethoate concentration at 25ºC; on the other hand, the highest dimethoate concentration exposure at 25ºC induced effects on this enzymatic activity. At 25ºC, after 14 days of recovery the decrease on GST activity for the lower concentration cannot be considered an inhibition response, but a basal value according to Ferreira et al. (2010). However, the value for the control showed a significant increase during the test, higher than basal values reported (Ferreira et al., 2010) and higher than values from organisms sampled

before the starting of the test for the same temperature. These differences observed for the control are unclear, since a return to basal levels were observed after 14 days of exposure.

An often reported effect of OPs is the induction of oxidative stress, by generating reactive oxygen species (ROS) as well as alterations within the antioxidant and scavenging system (Karami-Mohajeri and Abdollahi, 2011). The study carried out on the biomarkers GST, LPO and CAT showed also some degree of oxidative stress as a result of the exposure to dimethoate. LPO and CAT activity showed small responses to dimethoate exposure, although an increase on LPO was found at the 7 days of exposure at 25ºC (5x higher than control) that could be related to the high mortality observed. So, oxidative stress can be found as a result of dimethoate exposure during an adaptation period of 96h/7 days and also related to possible effects induced by the higher exposure temperature (25ºC). In fact, previous works have shown situations of oxidative stress for exposure to increment temperatures (e.g. Lesser, 2006; Lesser and Kruse, 2004), mainly leading to elevated dehydration (França et al., 2007). In addition CAT activity has been also reported to decrease as a result of the increase of superoxide anions (Velki and Hackenberger).

Energy response biomarker LDH apparently responded to dimethoate exposure at 20ºC, independently from concentration. After 7 days of exposure and 7 days of recovery this biomarker showed differences at the highest concentration under both temperatures. Although a clear response was not obtained by using this biomarker one can hypothesise that dimethoate may be interfering in the glycogen cycle and some of the effects found for this enzyme might also be associated with decreases in carbohydrates content (Moorthy et al., 1983).

Therefore, one main finding was that there were different responses for each energy reserve within each temperature of exposure. For lipids, an increase was observed in some of the time points and for both temperatures of exposure, but in general dimethoate did not seem to affect the lipid content. The carbohydrates presented a similar pattern of decrease for both temperatures within the first 7 days of exposure, after which the effects of dimethoate seemed to disappear and values tended to the basal levels. As expected, total protein did not present significant changes, as it is known that they are the last energy reserve to change or be used upon stress exposure. As in the previous case, an increase was also observed after

96 h exposure at 25ºC. Lower energy contents were observed mainly at 20ºC and for the lowest concentration. In general, energy reserves presented essential data to understand dimethoate toxicity even indicating a possible interaction within the two different temperatures. Whereas for the 20ºC exposure, almost no increase was found in the energy reserve content for the experimental duration, the 25ºC exposure presented not only a great content increase, but also around the 7/14 days of exposure these reserves reached a plateau, indicating an equilibrium in the organism physiology. In addition, it was also possible to depict small variations after reaching the plateau. The results obtained between the 96 h and 7days of exposure at 25ºC indicated an increase in carbohydrate, lipid and the protein content leading into a new plateau, which might also indicate some physiological or behavioural changes that the organisms underwent to prevent stress. In this case it seems also plausible to transpose all these results on the interaction between temperature and dimethoate into a disturbance in the moulting cycle. Along with the protein increase, it was also expected an increase in the other energy reserves as observed within our experiment. This impairment in the moulting cycle, which seems to happen when organisms are exposed to 25ºC, may also be a behaviour strategy to increase its feeding rate, so they could endure the stress for a longer time period. Previous works have already reported an increase in the muscle groups located in the anterior and posterior segments, which can be directly related to the protein content, and also that the moult, which is biphasic, and can occur with hours or days (Whiteley and El Haj, 1997). This could be that organisms exposed to 20ºC had a total protein content that did not increase in quantity, but stayed constant, and the increment in the energy reserves resulted from carbohydrate and lipid changes. These results are contradicted in several previous works where a decrease in feeding behaviour could be observed for terrestrial isopods exposed to stressors (Abdel-Lateif et al., 1998; Donker et al., 1998). Nevertheless one must consider that these studies have been performed with contaminated food, and although small amounts of pesticide must had been adsorbed by the leaves in our study, a possible avoidance was due to the effects on the organisms and not by changes in palatability.

Finally, in this study the available energy as a parameter did not seem to be a good indicator of the organisms' status, since little variation in contents was observed. The same happened with the CEA since differences in Ea values were low, and the organisms' behaviour

(represented by the Ec values, where higher consumption ratios relates to organisms' higher activities) were not intense enough to cause an impact.. Whenever a decline in CEA was observed, it was indicating either a reduction in available energy or a higher energy expenditure, both resulting in a lower amount of energy available for growth, reproduction or basal activity (de Coen and Janssen, 1997).

Within the two temperatures used, differences were observed in terms of the total available energy, energy consumption and subsequently the CEA. In fact the increase of Ec and decrease in Ea for the exposed organisms at 20ºC is contrary to the patterns shown at the 25ºC exposure, which may suggest that at 20ºC organisms did not slow their metabolism. However, at 20ºC, temperature did not seem to be the dominant factor influencing the energy-related parameters, but dimethoate, as can be seen by the patterns of Ea and Ec, as a result of the detoxification process. Contrary, for the organisms exposed to 25ºC, it did not seem that any of the stressors had a preponderance effect over the other, as organisms decreased significantly their Ec and allocated energy to the detoxification process. At this temperature, the Ea increase might be explained by an increase in isopods' feeding activities. This type of behaviour has not yet been reported and new studies regarding the combined effects of temperature and dimethoate on feeding rates would provide further information.

The recovery period did also provide some important and interesting remarks on organisms previously exposed to dimethoate. From previous works with other non-target organisms, such as earthworms, a slow recovery rate in AChE has also been reported (Aamodt et al., 2007). In the present work at 20ºC and at the highest dimethoate concentration, the AChE inhibition was in accordance with studies that show that it can become an irreversible process (Ranjbar et al., 2005). The organisms exposed to 20ºC followed a similar pattern when regarding oxidative stress biomarkers where a slow recovery was observed, although the LPO rates, that indicate damage from ROS, reached similar levels of those in the control after the end of the test period. For the organisms exposed to 25ºC although a similar pattern can be observed, the LPO rates continued to be significantly higher than the control, which may indicate that this temperature may influence the recovery of the organisms even after 14 days. The fluctuation of the energy related parameters in both the 20ºC and the 25ºC exposure are in accordance with results from biomarkers. In fact it can be hypothesised that the initial fluctuation (96 h/7 days) that could be seen in the exposure period which was more

pronounced in the recovery period, was a possible adaptation of organisms to reach homeostasis again.

All this supports the idea that a slow recovery is present, but also suggests an important highlight that these non-target organisms can continuously be affected by dimethoate even when exposed to recommended field doses under single applications. In a broader context the results obtained in this study can be summarized into three major points. First the impact of dimethoate in the main target enzyme AChE, and also in other oxidative stress biomarkers which are necessary to biotransform and handle the reactive oxygen species (ROS) within the organisms' body. Second the impact of temperature for the toxicity of dimethoate that combined with the pesticide may lead to higher toxicity, higher mortality rates and a decline in populations. Finally the sub-lethal effects observed when organisms were exposed to 0.4 mg dimethoate/kg soil, represented by changes in AChE, GST, energy consumption, CEA, can indicate the impairment of key functions essential for the maintenance of these organisms individually, and be transposed to the population level due to a possible effect on their reproduction behaviour/pattern.

5. Conclusions

As previously shown by other studies, dimethoate affected the enzyme AChE but also other biomarkers such as the detoxification enzyme, GST and the damage related biomarker, LPO. The increase of LPO also seemed to be related to the high mortality observed in 25ºC exposure.

The organisms exposed to the lower concentration of 0.4 mg dimethoate/kg soil, simulating a field application dose, presented low to moderate toxicity which was in accordance with the work of Fischer *et al.* (1997) and therefore was used as baseline for this study. However, in the organisms exposed to the highest concentration (10 mg dimethoate/kg soil), toxicity was mainly due to the inhibition of AChE and the degradation of dimethoate that can possibly lead to the formation of highly toxic metabolites and also reactive oxygen species and oxidative stress that caused high LPO rates.

This study showed an increase in the energy reserve contents within the first 7 days of exposure that could be linked to an impairment of the moult cycle or an increase in the feeding behaviour. This study highlights also a possible strategy of recovery for these organisms with an increase in total lipid content, which was the only energy reserve that returned to baseline levels within the recovery period.

Generally, long term experiments using realistic concentrations should be performed to understand the mechanisms of toxicity of stressors, such as dimethoate, and to develop a baseline for future studies. This will allow better understanding of the specific mechanisms underlying the toxicity process and detoxification pathways. In addition, the results from soil chemical analysis highlight the possibility that isopods can increase the decay rates of dimethoate, as also reported for other pesticides.

Acknowledgment

This study was supported by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia within the research project CLIMAFUN- CLImate Changes and Potential Impact on Soil FUNctional Ecology (FCOMP-01-0124-FEDER-008656) and through a PhD grant (SFRH/BD/65739/2009) awarded to Nuno G. C. Ferreira by FCT. The authors would like to thank for the technical support and advice given by Dr. Abel Ferreira and Dr. Ceri Morris.

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CHAPTER IV

Long-term exposure of the isopod Porcellionides pruinosus to nickel: costs in the energy budget and detoxification enzymes.

Long-term exposure of the isopod *Porcellionides pruinosus* to nickel: costs in the energy budget and detoxification enzymes.

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Abstract:

Terrestrial isopods from the species *Porcellionides pruinosus* were exposed to the maximum allowed nickel concentration in the Canadian framework guideline (50 mg Ni/kg soil) and to 5x this concentration (250 mg Ni/kg soil). The exposure lasted for 28 days and was followed by a recovery period of 14 days where organisms were changed to clean soil.

Organisms were sampled after 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, and 28 days of exposure, and at days 35 and 42 during the recovery period. For each sampling time the acetylcholinesterase (AChE), glutathione *S*-transferases (GST), catalase (CAT), lactate dehydrogenase (LDH) activities were determined as well as lipid peroxidation rate (LPO) along with lipids, carbohydrates, proteins content, energy available (Ea), energy consumption (Ec) and cellular energy allocation (CEA). The integrated biomarker response (IBR) was calculated for each sampling time as well as for each one of the above parameters. Mortality was also recorded throughout the assay.

The results obtained showed that nickel causes toxicity by inducing oxidative stress, evidenced by results on GST, GPx, CAT or LPO, but also on changes in the energy reserves content of these organisms. In addition, this study showed that these organisms possess a specific strategy to handle nickel toxicity. In this case, biomarkers were associated with costs in the energy budget, and the increase of energy reserves has a compensation for that cost.

***Keywords*:** integrated biomarker response, oxidative stress, metals, environmental risk assessment.

1. Introduction

In order to infer about the impact of stressors in natural ecosystems, ecotoxicological studies have been traditionally based on individual to population level effects, like mortality or reproduction. Nevertheless, it has been lately suggested that a full insight into these effects, with a comprehensive understanding of their causes, is only possible if different/lower levels of biological organization are assessed (Moore et al., 2004). The measurement of biomarkers at a sub-organismal level have already proved to be an effective tool to improve these procedures since they detect earlier signs of stress, possess more sensitivity than the traditional ecotoxicological tests, and show an accurate relationship between toxicant exposure and the biological response (Morgan et al., 1999). In addition they can provide crucial information on stressors' modes of action (MoA), which improves the knowledge on their related effects. Along with the measurement of biomarkers, the quantification of energy reserves and the metabolic costs associated to hand the stressor have already proved to be a good parameter to use in ecotoxicology (e.g. de Coen and Janssen, 1997; Vink and Kurniawati, 1996). In this way, the depletion of energy reserves can be probably associated with negative impacts in growth, reproduction and consequent impairment of population structure and dynamics (de Coen and Janssen, 2003).

Although being largely unknown, the soil compartment is one of the most important environments regarding structure, function, taxonomic diversity and trophic relations. In addition, and not less relevant, soils are crucial resources to man and its quality is dependent of its proper functioning. Soil functioning has been suggested to be hierarchically controlled by multiple natural factors, of which edaphic organisms constitute an important component (Lavelle, 1996). Nevertheless, by influencing each one of these functional levels, human activity can constitute the key factor affecting soil, frequently impairing these ecosystems' health (Lavelle, 1996). One of the most ubiquitous human-induced problems is the release of xenobiotics, particularly metals. The exposure to xenobiotics not only affect organisms, but may also change the overall soil functions, causing a decrease in soil quality and soil services (MEA, 2005).

Terrestrial isopods are macroinvertebrates involved in decomposition processes, vegetal litter fragmentation and re-cycling of nutrients (Ferreira et al., 2010; Loureiro et al., 2006;

Zimmer, 2002; Zimmer et al., 2003). The species *Porcellionides pruinosus* has been described as a good test-organism to evaluate soil contamination or changes in their habitat, and was therefore the one selected for the present study (Jansch et al., 2005; Loureiro et al., 2009; Loureiro et al., 2005; Takeda, 1980; Vink et al., 1995). Isopods have particular value when studying the stress caused by metals since they display exceptional environmental ion assimilation (Donker et al., 1990; Hopkin, 1990) together with a unique homeostatic metallo-sequestration processes in the hepatopancreas' cells (Morgan et al., 1999; Tarnawska et al., 2007). Both these processes display this group's ability to target individual metals to highly specific cell types and into specific intra-cellular compartments and constitute the main reason to show some of the highest internal metal concentrations among soil organisms (Heikens et al., 2001; Vijver et al., 2004). Given their position in the food chain, they can be considered critical regarding biomagnification of metals for higher trophic levels. Therefore studying how metals impair and role their homeostasis is of crucial importance. Another important feature present is their hard exoskeleton that prevents the metal exposure through direct absorption whilst placing the organism under physiological requirement for calcium to support the moult cycle (Vijver et al., 2005). Nevertheless, exposure routes include the pleopods structures that are responsible for the absorption of pore water from soil by capillary action (Sutton, 1980) or through the ingestion of soil particles or food (Diamond, 1999).

Nickel is a micro essential transitional metal that, although naturally occurring, can be found at higher levels due to anthropogenic activities (Phipps et al., 2002). Its effects on soil invertebrates is still very scarce, but overall it is considered a carcinogenic metal, that impacts gene transcription, translation processes and even the phosphate cycle (Lee et al., 1995; Pane et al., 2003; Vandenbrouck et al., 2009).

A set of biomarkers was assessed in order to include and study several pathways of stress: neurotoxicity (acetylcholinesterase), detoxification (glutathione *S*-transferases), oxidative stress (catalase, glutathione peroxidase, lipid peroxidation), energy related enzymes (lactate dehydrogenase) and energy reserves (lipids, carbohydrates and protein contents), along with energy related parameters such as energy consumption, energy available and cellular energy allocation. In addition, mortality was also recorded as a parameter transposing results from individuals to populations.

As previously stated, the use of terrestrial isopods in ecotoxicology is not new and considerable work has been already described, including different procedures and test durations, established for evaluating different endpoints (Calhôa et al., 2012; Loureiro et al., 2006; Loureiro et al., 2002; Morgado et al., 2013; Santos et al., 2010; Tourinho et al., 2013). Despite the duration required to evaluate different live trait parameters, the use of long term exposures are not frequently applied (Chapter III - Ferreira et al., 2015), although they are advisable if a more detailed and comprehensive effects of a stressor is aimed. In these cases it is possible to observe chemical's fate, bioavailability to organisms and even track possible time changes, along with recovery scenarios. Such long experiments are particularly relevant for metal toxicity given their inherent persistence and low mobility generally and possible chronic effects. For this reason a long-term assay was carried out to test the effects of nickel in the terrestrial isopod *Porcellionides pruinosus* during a 28 days exposure period followed by a 14 days recovery period, using the Integrated Biomarker Response to unify results/effects.

The results obtained will help to understand the process that undergoes exposure to nickel and posterior recovery by isopods, how are they coping with the need of reaching homeostasis and also to understand in a general way the pathways affected by the toxicity MoA of this metal to terrestrial isopods.

2. Materials and methods

2.1. Test Organism and Culture Procedure

Organisms from the species *Porcellionides pruinosus* Brandt (1833) that were previously collected from a horse manure heap and maintained for several generations in laboratory cultures were used as test organisms. Terrestrial isopods were fed *ad libitum* with alder leaves (*Alnus glutinosa*) and maintained at 22 ± 1°C, with a 16:8 h (light:dark) photoperiod. Culture's maintenance were performed twice a week, sprayed with water and food provided. Adult organisms (15 - 25 mg wet weight) were used in the experiments from both genders

and organisms with abnormalities, moulting characteristics or pregnant females were excluded.

2.2. Soil Spiking

Tests were performed using the natural soil LUFA 2.2 (LUFA, Speyer). The properties of this soil include a pH = 5.5 ± 0.2 (0.01 M $CaCl_2$), water holding capacity = 41.8 ± 3.0 (g/100 g), organic C = 1.77 ± 0.2 (%), nitrogen = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand.

Soil was spiked with nickel in the concentrations of 50 and 250 mg Ni/kg soil, with a final moisture content equivalent to 50% of the soil water holding capacity (WHC). The concentration of 50 mg Ni/kg soil represents the maximum concentration allowed by the Canadian framework guideline (CBP, 2010).

2.3. Experimental procedure

Exposures were performed in plastic boxes (26 length x 18 width x 7.5 height cm), containing approx. 2 cm layer height of Lufa 2.2 soil and 40 isopods (per box). Test organisms were collect from culture boxes, weighted and placed in each test-box. Food was supplied *ad libitum* (alder leaf disks - Ø 10 mm) in small quantities but throughout the test period whenever necessary. This allowed a continue food availability and a small soil coverage by leaves that could influence isopod's exposure to contaminated soil. Organisms were exposed to 50 and 250 mg Ni/kg soil in a 16:8 h (light:dark) photoperiod, at 20ºC. A total of five replicates were performed for each concentration. Four organism from each box/replicate were collected at time 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days (exposure period) and 35 days, 42 days (recovery period). In the results section, the 35 and 42 days of test duration will be denominated as 7 and 14 days of post-exposure.

The enzymatic biomarkers glutathione *S*-transferases (GST), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LPO) were measured using a pool of two full-body organisms per replicate. From another organism, the head was separated and used to analyse acetylcholinesterase (AChE), corresponding to one replicate. All chemicals used in these experiments were obtained from Sigma-Aldrich Europe, except the Bradford reagent, which was purchased from Bio-Rad (Germany), and hydrogen peroxide from Fluka.

For the energy related parameters (total lipids, carbohydrates and proteins and electron transport system), only one organism was used as a replicate. At each sampling time, the number of dead organisms were counted and removed from the test boxes.

2.4. Biochemical biomarkers, energy reserves and IBR

The protocol used is extensively described in the supplementary data, and was previously described by Ferreira et al. (2010) and Ferreira et al. (2015). The lipid peroxidation (LPO) assay was adapted on the methods described by Bird and Draper (1984) and Ohkawa et al. (1979) and adapted to microplate format. The other oxidative stress related biomarkers glutathione *S*-transferases (GST), glutathione peroxidase (GPx) and catalase (CAT) activity were determined based on the method described Habig et al. (1974), Mohandas et al. (1984) and Clairborne (1985) respectively. The acetylcholinesterase (AChE) activity was performed according to the Ellman method (Ellman et al., 1961) adapted to microplate format by Guilhermino et al. (1996). For all biomarkers, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

To determine total protein, carbohydrate and lipid contents, energy consumption (Ec), energy available (Ea) and cellular energy allocation (CEA), the protocol was adapted from the one described for daphnids from de Coen and Janssen (1997). The Ea, Ec and CEA values were calculated as described by Verslycke et al. (2004):

Ea (available energy) = carbohydrates + lipids + proteins (mJ / mg org.)
Ec (energy consumption) = ETS activity (mJ / mg org. / h)
CEA (cellular energy allocation) = Ea/Ec (/h)

To integrate all results from the different biomarkers and understand global/general responses, the integrated biomarker response (IBR) was calculated according to Beliaeff and Burgeot (2002) which is also extensively described in the supplementary data. The IBR calculations were always performed with the same order of parameters for all sampling times: the neurotoxicity biomarker AChE (the enzyme for which the pesticide was designed to inhibit), followed by the detoxification and oxidative stress biomarkers LPO, GST, GPx and CAT, and finally the lipids, carbohydrates and proteins content, the energy available (Ea), the energy consumption (Ec) and the CEA that integrates the last two parameters. Further details can also be found in the supplementary data.

2.5. Data Analysis

To compare differences between treatments the one-way analysis of variance (ANOVA) was performed followed by a Dunnett's comparison test in order to discriminate statistically different treatments from the control (SPSS 1999). Whenever necessary data transformation was used to achieve normality, however when data did not show a normal distribution or homoscedasticity, the non-parametric test Kruskal-Wallis One Way Analysis of Variance on Ranks was used.

Data values that were higher or lower than the mean value, plus or minus two times the standard deviation, were considered outliers, and withdrawn from analysis (Rousseeuw and Croux, 1993). Whenever there was enough data ($n \geq 3$, due to high mortality rates), a two-way analysis of variance (two-way ANOVA) was performed to check for interactions between time and concentration. The two-way ANOVA was performed separately for the exposure and the recovery period. The one-way ANOVA and two-way ANOVA with significance of $\alpha = 0.05$. Due to the mortality observed, the last sampling time (14 days of recovery) was excluded from the analysis.

3. Results

In Fig. 4.1 is presented the number of dead organisms by the cumulative number at a given sampling time. Due to high mortality observed at 14 days of recovery, no sampling could be performed.

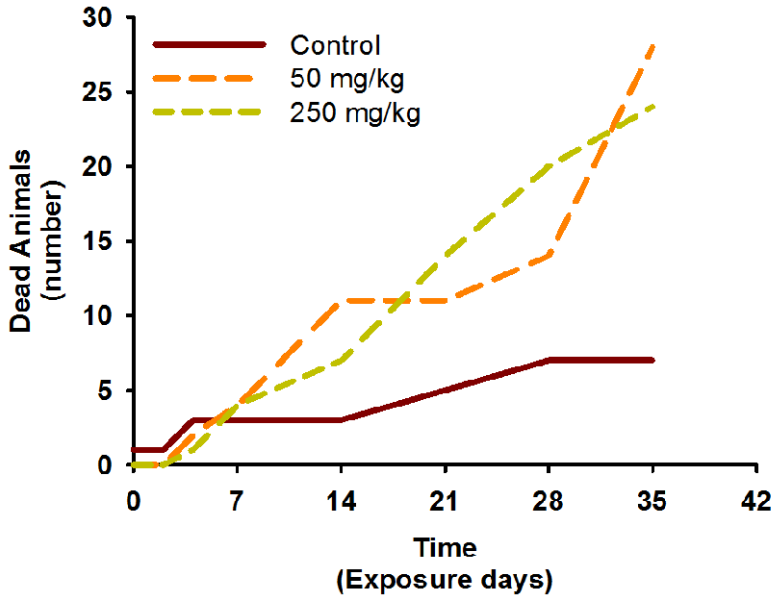

Fig. 4.1 Cumulative total number of dead organism from the species *Porcellionides pruinosus* during the exposure and recovery period in the control and exposed to 50 mg Ni/ kg soil and 250mg Ni/ kg soil. A total of 200 organisms were exposed per treatment.

3.1.Enzymatic biomarkers activity

The detailed biomarkers activity and energy reserves content of organisms exposed to nickel during the exposure and recovery periods are presented in Fig 1SD (supplementary data).

The neurotoxicity biomarker AChE showed a significant increase for the highest concentration after 14 days of exposure (One Way ANOVA, $F_{2,11} = 4.557$; Dunnett's test $p = 0.036$). For the oxidative stress related biomarkers significant increases were also observed for LPO (highest concentration, 28 days of exposure; One Way ANOVA, *reciprocal*

transformation, $F_{2,10} = 7.810$; Dunnett's test $p = 0.009$), for GST (highest concentration, 7 days of recovery; One Way ANOVA, $F_{2,10} = 7.561$; Dunnett's test $p = 0.010$). The biomarker CAT showed a significant increase at the highest concentration after 24h of exposure (One Way ANOVA, $F_{2,9} = 16.301$; Dunnett's test $p = 0.001$) and a significant decrease after 7 days of recovery (One Way ANOVA, *reciprocal transformation*, $F_{2,9} = 9.392$; Dunnett's test $p = 0.019$).

For the energy related parameters significant increases were observed for carbohydrates for the lowest concentration after 24h of exposure (One Way ANOVA, $F_{2,10} = 21.160$; Dunnett's test $p < 0.001$) and for the highest concentration after 96 h of exposure (One Way ANOVA, $F_{2,11} = 5.096$; Dunnett's test $p = 0.027$). The other parameter that also showed significant differences was Ec, where a significant decreases were observed after 24 h, 7 and 28 days of exposure for the lower concentration (respectively ANOVA on Ranks, $H = 6.806$, d.f. = 2; Dunn's test $p = 0.033$; One Way ANOVA, $F_{2,11} = 10.681$; Dunnett's test $p = 0.003$; One Way ANOVA, $F_{2,9} = 7.013$; Dunnett's test $p = 0.015$). Significant increases were also observed for Ec for both exposure concentrations after 96 h of exposure (One Way ANOVA, $F_{2,10} = 8.800$; Dunnett's test $p = 0.006$) and for the higher concentration after 7 days of recovery (One Way ANOVA, $F_{2,11} = 7.6586$; Dunnett's test $p = 0.011$).

Interactions between time of exposure and nickel concentrations were significant in the exposure period only for catalase (Two Way ANOVA, $F_{2,83} = 2.006$; $p = 0.027$). In the recovery period significant interactions were observed for: GST (Two Way ANOVA, $F_{2,21} = 5.949$; $p = 0.009$), GPx (Two Way ANOVA, $F_{4,24} = 2.237$; $p = 0.012$), Ec (Two Way ANOVA, $F_{2,18} = 9.924$; $p = 0.001$) and lipids (Two Way ANOVA, *reciprocal transformation*, $F_{2,20} = 3.634$; $p = 0.045$).

3.2. Integrated Biomarkers response (IBR)

The IBR starplots are presented in Fig. 4.2 and Fig. 4.3. They include the scores of each parameter and each sampling time during the exposure and recovery period. Better or worse

scores (respectively lower or higher values) obtained for each parameter are summarized in Table 1SD (supplementary data).

The analysis of the IBR values according to sampling time (Fig. 4.4) showed frequently worse scores (higher values) for the control rather than the Ni treatments (the exceptions are 24 h, 96 h, 28 days of exposure and 7 days of recovery). It also shows that frequently the lower concentration had better values (lower values) than the higher concentration of exposure. The sampling time that reports to the recovery period (7 days of recovery) was the only one showing an expected result with values increasing with the increasing of concentrations.

Looking at the IBR values according to the measured parameter (Fig. 4.5), the results showed a different scenario. Control presented the better or second better values (lower values) for all biomarkers except CAT. On the contrary for the energy related parameters control presented an opposite trend.

A comparison between the mean IBR/n values for all sampling times did not show any statistically different from the control, regarding the exposure concentrations (One Way ANOVA, $F_{2,26} = 0.182$; $p = 0.835$).

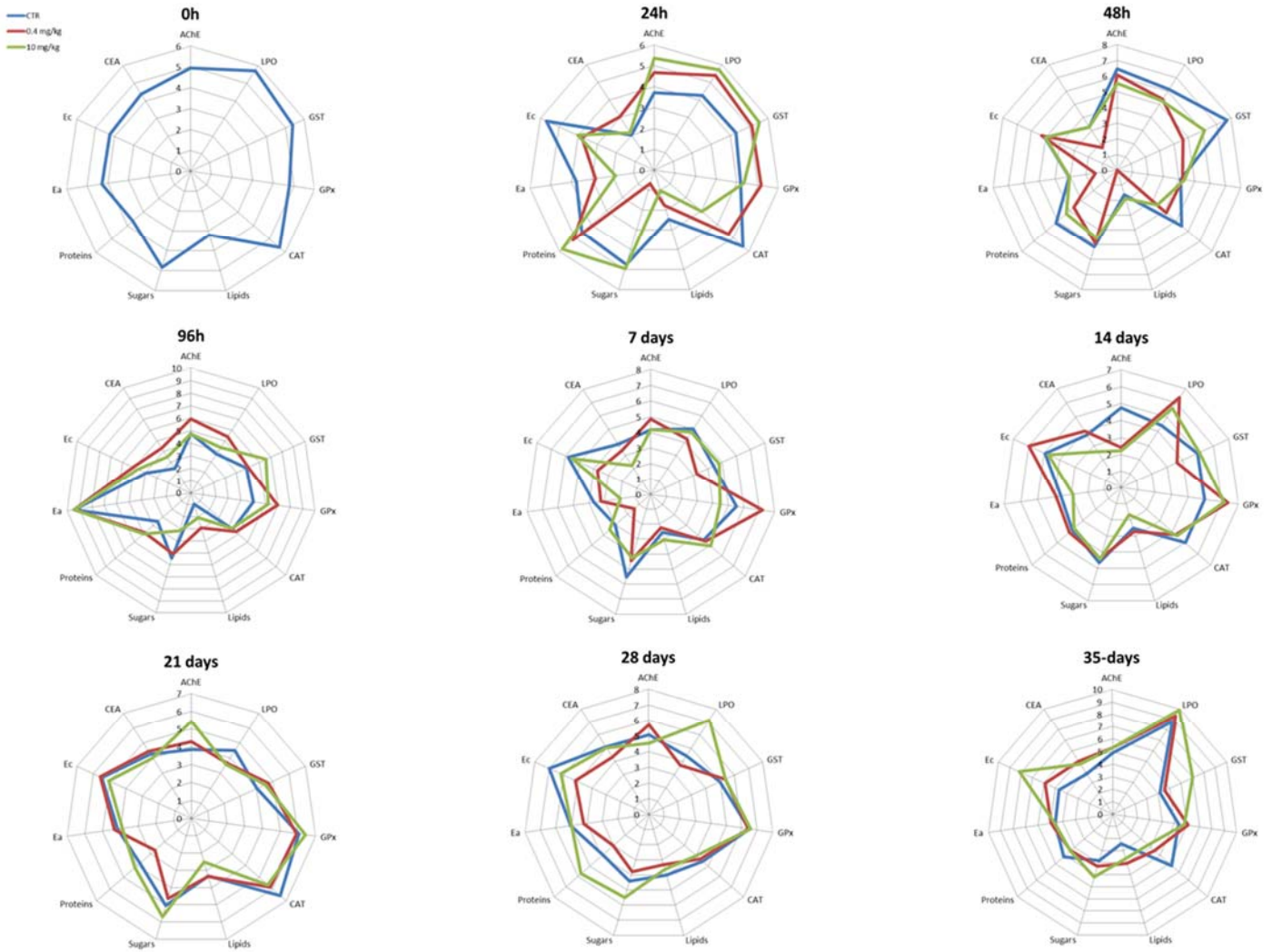

Fig. 4.2 Star plots for each sampling time of *Porcellionides pruinosus* exposed to 50 mg and 250 mg Ni/kg soil (exposure period: 0h, 24h, 48h, 96h, 7-days, 14-days, 21-days, 28-days; recovery period: 35-days, 42-days). AChE = acetylcholinesterase, LPO = lipid peroxidation, GST = glutathione *S*-transferases, GPx = glutathione peroxidase, CAT = catalase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

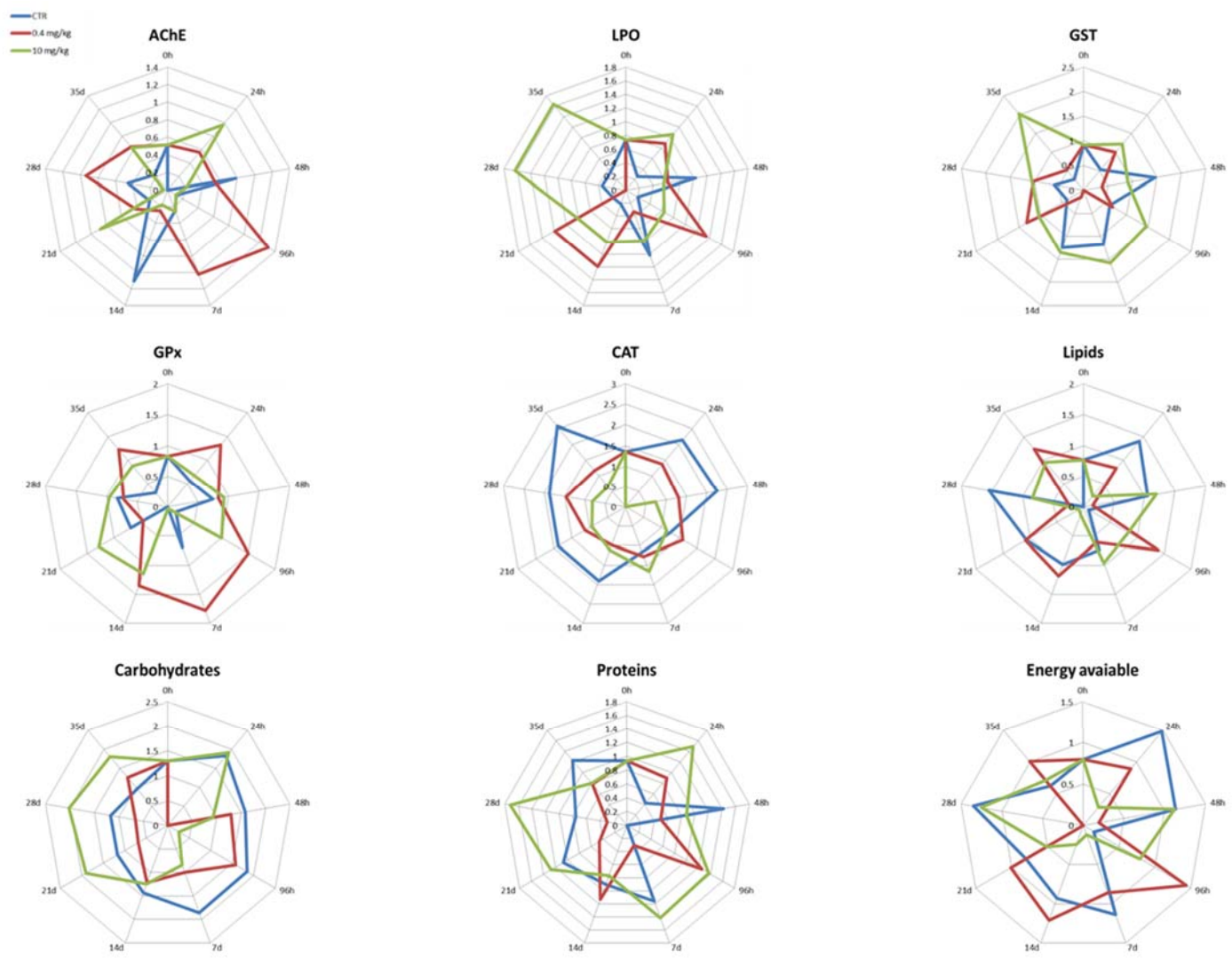

Fig. 4.3 Integrated Biomarker Response of *Porcellionides pruinosus* in control and exposed to nickel (50 and 250 mg/kg soil) during the exposure and recovery period. AChE = acetylcholinesterase, LPO = lipid peroxidation, GST = glutathione *S*-transferases, GPx = glutathione peroxidase, CAT = catalase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

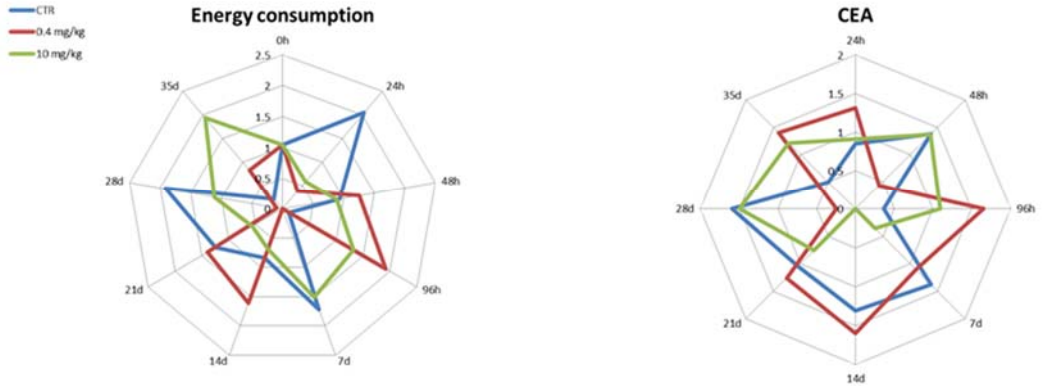

Fig. 4.3 (cont.) Integrated Biomarker Response of *Porcellionides pruinosus* in control and exposed to nickel (50 and 250 mg/kg soil) during the exposure and recovery period. AChE = acetylcholinesterase, LPO = lipid peroxidation, GST = glutathione *S*-transferases, GPx = glutathione peroxidase, CAT = catalase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

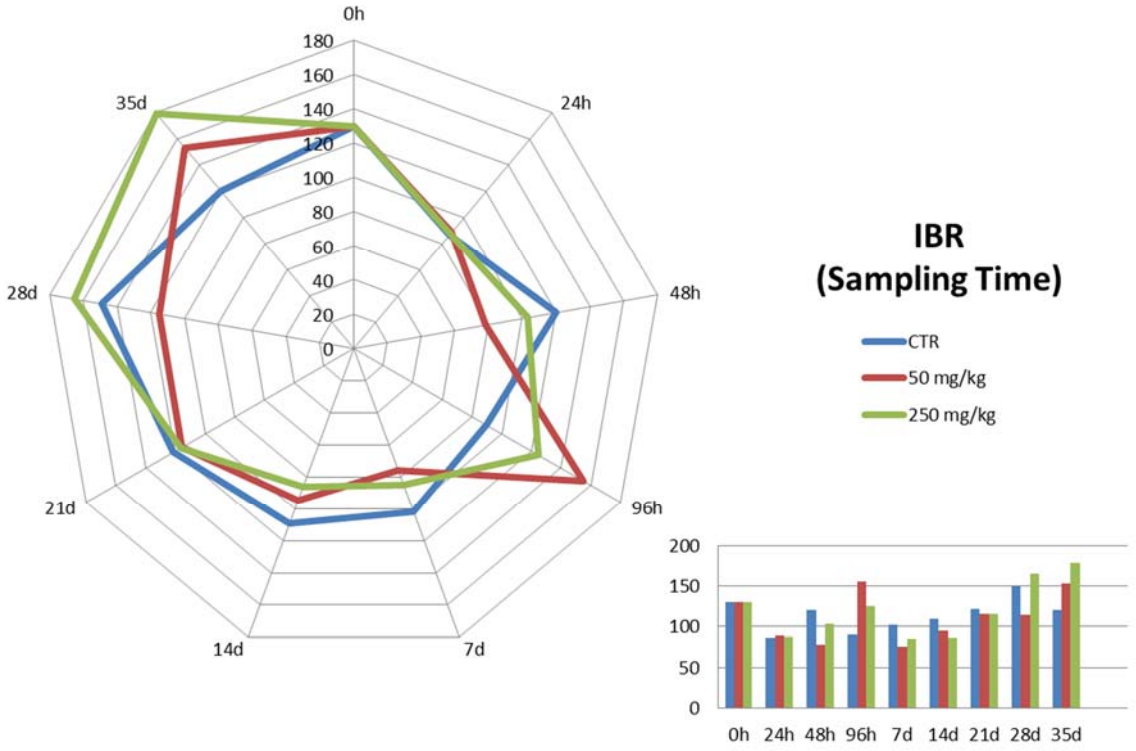

Fig. 4.4 Integrated Biomarker Response (IBR) of *Porcellionides pruinosus* in the control and exposed to nickel (50 and 250 mg/kg soil). Exposure period: 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days.

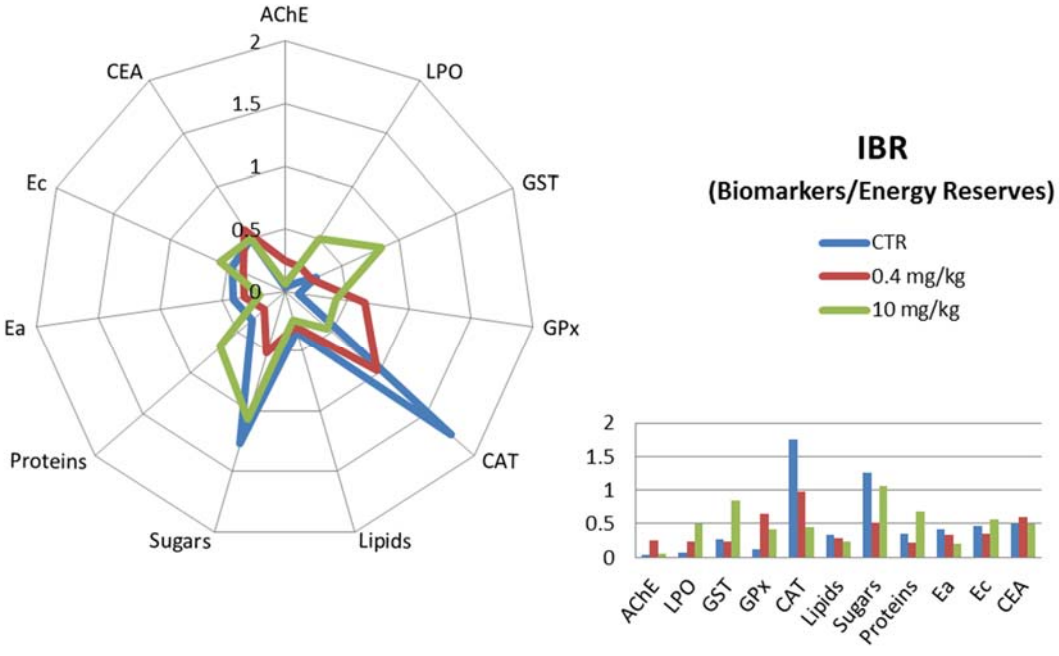

Fig. 4.5 Integrated Biomarker Response (IBR) of *Porcellionides pruinosus* in the control and exposed to nickel (50 and 250 mg/kg soil) for each measured endpoint. AChE = acetylcholinesterase, LPO = lipid peroxidation, GST = glutathione *S*-transferases, GPx = glutathione peroxidase, CAT = catalase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

4. Discussion

In the present study the mechanisms involved in nickel toxicity to the terrestrial isopod *P. pruinosus* were assessed. This analysis, not only provided information about the toxicity inherent to the nickel concentrations used, but also the patterns observed at the different sampling times, and the differences between the exposure and recovery period.

The first highlight from the present study was a possibly increase on mortality when this long term exposure to nickel was carried out, especially at the highest concentration. But we should regard this carefully as we cannot state accurately a mortality rate as organisms were sampled in several points in time. Even though, at the end of the test 20 organisms were recorded dead at the highest concentration (250 mg Ni/kg), when comparing to the 7 dead organisms from the control. In a 28 day study with the earthworm *Eisenia veneta*, nickel induced a reduction of 10% on the cocoon production at 85 mg Ni/kg soil, and adult survival was only reduced at concentrations above 245 mg Ni/kg soil (Scott-Fordsmand et al., 1998). In another study with *Folsomia candida*, the LC50 for nickel exposure was 279 mg Ni /kg dry soil (Broerse and van Gestel, 2010).

When looking at the biomarkers and energy reserves data, two main results can be highlighted: 1) the IBR analysis showed that the control was presented worse values than the exposure concentrations in some of the sampling times; 2) contrasting results were obtained when looking individually at each biomarker or energy-related parameters where organisms from the control showed to be in better conditions than treatments for the measured biomarkers, but in worse conditions regarding energy related parameters.

In order to better understand the previous results, a general characterization of the effects of metals in organisms is necessary. In several studies, the exposure to low concentrations of metals lead to hormetic responses (e.g. Calabrese and Baldwin 2001; Calabrese and Blain 2005; Lefcort et al. 2008; Nascarella et al. 2003). If the same hormetic response was present, we would expect that the higher concentration presented higher toxicity values (higher IBR values) and the lower concentration lower toxicity values than our control, which is not the case. For this reason, the most plausible explanation would be that the higher concentration used in this study (250 mg/kg soil) can still not be considered high enough to entail serious

stress to these organisms. Also, the mortality observed showed that both nickel concentrations used caused more mortality than the one observed for the control, rejecting any hormetic response, and supporting an increase of toxicity with the increase of nickel concentration.

Although a complete answer cannot be derived from the previous observation, the analysis of the IBR values for each measured parameter showed different patterns between biomarkers and energy related parameters. The majority of the measured biomarkers showed that nickel exposures were inducing more toxicity than the control, and lipids, carbohydrates and proteins were always lower in the control.

Regarding biomarkers, it is possible to observe that nickel affected oxidative stress biomarkers as expected for metals, but could also target the neurotoxicity pathway signalised by the biomarker AChE. Although an inhibition of AChE would be expected when organisms are exposed to stressors (e.g. Ferreira et al. 2015; Ribeiro et al. 1999; Stanek et al. 2006), a significant increase was observed (14 days of exposure). This has been also reported for the same species under stress exposures (Morgado et al., 2013; Santos et al., 2010).

Nevertheless results should still be analysed with caution as in a previous study, where Frasco et al. (2005) evaluated the activity of AChE in vitro, after exposure to five metals (nickel, copper, zinc, cadmium and mercury) showed that with the exception of nickel, all other metals inhibited AChE. This study also showed that the technique used to measure AChE activity can strongly influence the obtained results, since complexes between the metals and the reaction buffers were formed. Although the previous study was based on *in vitro* techniques, the results obtained in our study should not be discarded or observed has an artefact.

Regarding the oxidative stress biomarkers (LPO, GST, GPx and CAT), the results showed a clear connection between each other. The exposure of isopods to both nickel concentrations showed an increase in LPO rates with the increase of nickel concentrations, which indicates that cells are being damaged. In the review of Valko et al. (2005) metals such as iron, copper, cadmium, chromium, mercury, vanadium and nickel had the ability to produce reactive

radical species (that include oxygen-, carbon-, sulphur- radicals) that may result, between other effects, on lipid peroxidation. These effects observed for LPO in this study were also reported in human lymphocytes *in vitro*, where a concentration-level dependency of lipid peroxidation was observed (Chen et al., 2003 in Valko et al., 2005). Likewise, a concentration-dependency and time-dependency was observed for plasma of human blood *in vitro* (Chen et al., 2002 in Valko et al., 2005) or even in the intestinal mucosa of broilers fed with corn-soybean contaminated diet (Wu et al., 2013). The activity of the other biomarkers (GST, GPx and CAT) apparently followed a pattern within the oxidative stress detoxification pathway. For example, whenever GST and GPx are inhibited, to deal with the oxidative stress, CAT activities are induced. This response within biomarkers can be seen using the IBR values, although when looking at them isolated, low or no changes can be depicted just by comparing to the control. Some of them suggested the oxidative stress process as being possibly important for the induction of carcinogenesis by nickel, despite a direct correlation between its ability to produce oxidative stress and its carcinogenicity is yet to be proved. Moreover, several other metals, such as copper and iron, are known to strongly induce oxidative stress but were not found to be carcinogenic (Gilman, 1962 in Valko et al., 2005). In other studies, Wu et al. (2013) similar results were reported, with inhibitions of GST, GPx and CAT in the intestinal mucosa of broilers, while in the study of Sunderman et al. (1985) no effects on CAT or GPx activities were observed in rats' blood. Although GST and GPx appeared inhibited and could be related to the GSH depletion effect already reported for nickel (Valko et al., 2005), it also sems that Ni can directly inhibit this enzymes since the assay to determine its activity is performed with high amounts of GSH does not being the cause of the inhibition observed. The enzymes GST and GPx are known to be strongly dependent of glutathione (GSH) for properly performing their functions. For instance, GPx transforms GSH in GSSG, a process necessary to handle the transformation of H_2O_2 into H_2O and O_2 (Cadenas and Davies, 2000). Similarly GST depends on the presence of GSH in order to act as catalysts for the conjugation of various electrophilic compounds (Armstrong, 1987; Gulick and Fahl, 1995).

The changes on the energy-related parameters are, as stated before, the endpoints that contribute the most for the control to show higher IBR values than the nickel exposures. However, the higher amounts of energy reserves observed in exposed organisms during most

of the sampling times were not expected. In fact, they were mainly expected at 96 h and 7 days of exposure, period where organisms normally show moulting processes (Ferreira et al., 2015), although other physiological processes could also be involved as described below. Previous studies suggest that nickel replaces iron in the oxygen carrier, switching the signal to permanent hypoxia, which in turn activates the hypoxia-inducible factor 1alpha (HIF-1) factor (Goldberg et al., 1988 in Valko et al., 2005). The HIF-1 is also involved in the regulation of several genes that belong to the glucose transport and glycolysis pathways (Semenza, 1999 in Valko et al., 2005). In addition, as described previously, isopods have unique processes to handle metals that range from metallo-sequestration in the hepatopancreas cells, to structural characteristics, such as the hard exoskeleton that limits the exposure route of metals. The former process involves a daily cycle of cells present in the hepatopancreas. There are two groups of cells: the large and binucleate '*B*' cells and the small uninucleate '*S*' cells. The '*S*' cells contain Cu-thiol vesicles whereas '*B*' cells accumulate Fe rich phosphate granules with a still unknown function. The '*B*' cells undergo a striking diurnal cycle of ultrastructural changes in which the contents of the apical cytoplasm are voided in an apocrine manner over ~11 hours, followed by a recovery phase, during which the cytoplasm is recharged with lipids, glycogen and Fe rich inclusions (Hames and Hopkin, 1991). In our study, the collection of organisms was always performed within the same timeline, which excluded the variation of sampling organisms in different periods of the daily cycle. Nevertheless the mechanism involved on the regulation of metals may also be affected and such changes may be the reason for this pattern of high contents of energy reserves. Despite the processes involved, the Ea showed a clear pattern of dose-dependency, where organisms exposed to higher doses of nickel appeared to accumulate more energy reserves than the others from the control. This interesting result, along with a clear strategy of the organism, in order to handle nickel by modifying their behaviour (organisms exposed to the lower concentration showed lowed Ec, whereas the organisms exposed to the higher concentration showed higher Ec), led to CEA values very close to those found in the control. These results can then be supported in some degree by field studies, where populations of terrestrial isopods survive in areas with overall concentrations of nickel much higher than the used in this study (e.g. Alikhan and Storch, 1990; Bayley et al., 1997). In the work of Vandenbrouck et al. (2009) daphnids exposed to Ni, showed a decrease in the energy reserves content along with a decrease in Ec. Although a direct

comparison cannot be made, after 96 h of exposure the same decrease was observed for isopods, with higher IBR values for total lipids and proteins, but not for carbohydrates or Ec.

In the present study the exposure period was followed by a recovery period, where only few organisms were available due to the mortality observed during exposure. Anyway, in the first 7 days of recovery it was possible to observe retrieval from organisms. Although some stress could be observed after 7 days that might be confirmed for a 14 days recovery period, with the results obtained for LPO, which showed higher rates at 28 days of exposure, and identical levels in the recovery comes as an evident of this recovery. Once again this fast recovery comes in accordance with the previous studies stated before, that show the terrestrial isopods as species capable of handling environments with high metal contents.

5. Conclusions

Nickel is a commonly used metal that reaches the environment not only through natural sources but mainly from anthropological sources such as mining, alloy production, electroplating, refining or even welding. The effects of nickel in organisms, especially invertebrates are still pretty unknown and this type of studies can bring new insights. The evaluation of several biomarkers and energy-related parameters allowed to confirm oxidative stress as a cause of nickel toxicity and also brought new insights on energy budget regulation.

The organisms exposed to the lower concentration (50 mg Ni/kg soil), corresponding to the maximum allowed concentration in fields by the Canadian framework guidelines, generally presented low toxicity, with some biomarkers presenting conditions very similar to the control. A low toxicity was also experienced by the organisms exposed to the highest nickel concentration (250 mg Ni/kg soil), being however slightly higher than the lower concentration. In general both concentrations presented almost no variation in all the measured parameters when compared to the control.

The inclusion of a recovery period in ecotoxicological studies may be considered very useful regarding the information provided under long-term exposures that may not always cease when organisms are transferred to control conditions after an exposure period.

Although new information was added to previous studies, there is still the need to observe the effects of nickel at a molecular and metabolic level. In the isopods particular case, due to their capacity of living and tolerate environments with high concentrations of nickel, the proposed evaluation will provide even more insight on their regulation mechanisms.

Acknowledgment

This study was supported by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia within the research project CLIMAFUN- CLImate Changes and Potential Impact on Soil FUNctional Ecology (FCOMP-01-0124-FEDER-008656) and through a PhD grant (SFRH/BD/65739/2009) awarded to Nuno G. C. Ferreira by FCT. The authors would like to thank for the technical support and advice given by Dr. Abel Ferreira.

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CHAPTER V

Metabolic responses of the isopod Porcellionides pruinosus to nickel and dimethoate exposure assessed by ^{1}H NMR spectroscopy.

Metabolic responses of the isopod *Porcellionides pruinosus* to nickel and dimethoate exposure assessed by 1H NMR spectroscopy.

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Abstract:

Terrestrial isopods from the species *Porcellionides pruinosus* were exposed to soil contaminated with relevant concentrations of the metal nickel or the pesticide dimethoate. Prior to the exposure and after a period of 4, 7 and 14 days of exposure, animal tissue extracts were analysed by high resolution proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, in order to characterize their metabolic profiles and reveal the changes induced by each contaminant. The NMR analysis of the extracts gave characteristic low molecular weight metabolite fingerprints for this species, which had not been previously described. Moreover, compared to the metabolic profiles reported for other isopod species, over 20 endogenous metabolites were newly identified. The metabolic changes upon nickel or dimethoate exposure were then characterised by assessing the quantitative variations in specific metabolite levels (e.g. amino acids, sugars, lipids, etc.). The results showed that both contaminants caused dose- and time-dependent changes in the isopods' metabolome. Exposure to nickel was suggested to affect growth, moult and haemocyanin synthesis, along with glutathione biosynthesis, energy metabolic pathways and osmoregulation. Moreover, short-term exposure to this metal caused significant changes in lipids, possibly in relation to the role of hepatopancreas '*B*' cells in detoxification processes. In regard to dimethoate-induced variations, they possibly reflected moulting-related changes, disturbances in osmoregulation and energy metabolism and, additionally, disturbances on neurotransmission. Interestingly, the impact on lipids and choline-containing compounds was much smaller than in the case of Ni exposure.

***Keywords*:** pollution indicators, metabolic profile, NMR spectroscopy, pesticides, metals, environmental risk assessment.

1. Introduction

While traditional tests in ecotoxicology provide important information about the general impact on the individual (e.g. survival or reproduction) and the population, the thorough understanding of how a contaminant affects an organism requires the study of different levels of biological organization. Metabolic profiling (metabolomics) approaches enable alterations in the cellular metabolome (i.e. inventory of endogenous small molecules < 1 kDa) to be monitored, potentially revealing marker signatures of exposure and providing new insights on induced biochemical events (Moore et al., 2004). Even though the use of metabolomics in environmental sciences is still scarce, some studies have addressed the changes in the metabolic profile of some key model species (e.g. Guo et al., 2009; Liu et al., 2011; Wu and Wang, 2010). Regarding soil ecotoxicology, most studies are related to earthworms (e.g. Vale et al., 2003; Yuk et al., 2013), and, to our knowledge, there is only one report on terrestrial isopods, which presents metabolite fingerprints for the species *Porcellio scaber* and *Oniscus asellus* (Gibb et al., 1997), in a healthy (non-exposed) condition.

Terrestrial isopods are macro-invertebrates involved in decomposition processes, vegetal litter fragmentation and re-cycling process of nutrients (Ferreira et al., 2010; Loureiro et al., 2006; Zimmer, 2002; Zimmer et al., 2003), therefore being essential to maintain the function and structure of the soil compartment. Exposure to xenobiotics may affect edaphic organisms, consequently changing the overall soil function, and decreasing soil quality and soil services (MEA, 2005). The species *Porcellionides pruinosus* has been described as a good test-organism to evaluate soil contamination or changes in their habitat (Jansch et al., 2005; Loureiro et al., 2009; Loureiro et al., 2005; Takeda, 1980; Vink et al., 1995) and several endpoints, from the individual to lower organizational levels, have been used to evaluate the effects caused by different stressors. However, there is still the need to integrate the different organizational levels, in order to relate impairments at higher levels to molecular initiating events at lower levels.

Considering the above mentioned, the general aims of this study were: i) to characterize the metabolic composition of the terrestrial isopod *Porcellionides pruinosus*, thus allowing intra- and inter-species comparisons; ii) to understand how chemical exposure would change the metabolic profile of this species. For that purpose, two model stressors - the metal nickel and the pesticide dimethoate - were selected, based on their mode of action, and also on previous studies where information on effects at the individual level, enzymatic processes and energy reserves were assessed (Chapters III and IV). Terrestrial isopods were exposed to nickel or dimethoate for up to 14 days, and their metabolomic profile (measured by ^{1}H-NMR spectroscopy) compared to that of non-exposed animals (used as controls).

2. Materials and methods

2.1. Test Organism and Culture Procedure

Organisms used in this assay belong to the species *Porcellionides pruinosus* Brandt (1833), and were previously collected from a horse manure heap and maintained for several generations in laboratory cultures. In culture, isopods were fed *ad libitum* with alder leaves (*Alnus glutinosa*) and maintained at 22 ± 1°C, with a 16:8 h (light:dark) photoperiod. Twice a week cultures were water sprayed and food was provided. Only adult organisms (15 - 25 mg wet weight) were used in the experiments and no distinction between genders was made, although pregnant females were excluded. Organisms with abnormalities or moulting characteristics were also excluded from the trials.

2.2. Soil Spiking

LUFA 2.2 soil (LUFA-Speyer 2.2, Germany) is a sandy loam and was used for the exposures. This soil presented a total organic carbon content of 1.77 ± 0.2%, a pH (0.01 M $CaCl_2$) of 5.5 ± 0.2, nitrogen = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand and a water-holding capacity (WHC) of 41.8 ± 3% (g/100g). Soil

was spiked with nickel at concentrations of 50 and 250 mg Ni/kg soil, with a final moisture content equivalent to 50% of the soil WHC. The concentration of 50 mg Ni/kg soil represented the maximum concentration allowed by the Canadian framework guideline (CBP, 2010). For the dimethoate study, the two concentrations used were 0.4 and 10 mg dimethoate/kg soil, where the lowest concentration represents the recommended field dose after dimethoate application and the 10 mg dimethoate/kg soil refers to a concentration below the EC50 value found for the isopod *Porcellio scaber* in the work of Fischer et al. (1997) in LUFA 2.2 soil (EC50s for growth of 17.5 mg dimethoate /kg soil; for mancae / surviving female, 16.8 mg dimethoate /kg soil; for pregnant/surviving females, 15.4 mg dimethoate /kg soil).

2.3. Exposure experiments

Toxicity tests were performed in plastic boxes (26 length x 18 width x 7.5 height cm), containing approx. 2cm height of LUFA 2.2 soil layer, with 40 isopods per box. Alder leaf disks (Ø 10 mm, ± 20 mg) were supplied as food, using a quantity that prevented organisms to remain on top and avoid contaminated soil.

Organisms were exposed to control soil, 50 mg and 250 mg Ni /kg soil, 0.4 mg and 10 mg of dimethoate/kg soil, in a 16:8 h (light:dark) photoperiod, at 20ºC, for 14 days. During this period, organisms were sampled at four time points: prior to the exposure, 96 h, 7 days and 14 days after exposure. In each sampling time, and for each treatment, three replicates composed of 6 organisms each were collected.

2.4. Sample preparation and NMR data acquisition

Organisms were weighted and stored at -80 °C for a period no longer than one month. Before analysis, each sample (6 organisms per replicate) was homogenized using a sonicator (Kika Labortechnik, V200Scontrol, Germany) in 600 μL of K-Phosphate/D_2O 0.1M buffer, pH 7.0

and centrifuged (10 000rpm, 10 min, 4ºC). Then, 400 µL of supernatant were transferred into a 5 mm NMR tube to which 100 µL of D_2O (to provide a lock signal) containing 0.1% TSP-d4 (used for shimming) were added.

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer operating at 500.13 MHz for ^1H observation at 300 K. Standard 1D spectra with water presaturation (pulse program 'noesypr1d', Bruker library) were acquired with a 6510 Hz spectral width, 32 K data points, a 2 s relaxation delay (d1), 100 ms mixing time (d8) and 256 scans.

2.5. NMR data processing & statistical analysis

All 1D spectra were processed with a 0.3 Hz line broadening, zero filling to 64 K data points, manual phasing and baseline correction. The chemical shifts were referenced internally to the glucose signal at δ 5.23 ppm. Probabilistic quotient normalization (PQN - Dieterle et al., 2006) was applied (MATLAB version 7.12.0, The MathWorks Inc.) to account for dilution-independent effects on spectral area and enable quantitative comparisons. Spectral assignment was based on 2D total correlation spectroscopy (TOCSY) spectra and consultation of spectral databases (Bruker Biorefcode database and the human metabolome database (HMDB - Wishart et al., 2009). To evaluate metabolite quantitative variations, selected signals in the 1D spectra were integrated using Amix-Viewer (version 3.9.14, BrukerBiospin, Rheinstetten), and normalized by the PQN quotient of each spectrum. The resulting data was plotted into a heat map using the GENE-E software (http://www.broadinstitute.org/cancer/software/GENE-E/index.html). This software was also used to perform hierarchical cluster analysis of the metabolic profile obtained for different exposure concentrations and time points. The cluster analysis was performed using a Pearson Correlation Coefficient with Single Linkage.

A one-way analysis of variance (ANOVA) was performed to compare differences between treatments at each sampling time and Dunnett's comparison test was carried out to discriminate statistically different treatments from the control (SPSS 1999). When possible, data transformation was used to achieve normality. When data did not show a normal distribution, the non-parametric test Kruskal-Wallis One Way Analysis of Variance on Ranks was used. A Two Way ANOVA was also performed in order to infer possible interactions between time and concentration. The One Way and Two Way ANOVA used an $\alpha = 0.05$ significance.

3. Results

3.1. Metabolic profile of the species *P. pruinosus*

A typical ^{1}H-NMR spectrum of an isopod extract can be seen in Fig. 5.1, where a multitude of signals is detected, reflecting the complex rich composition of the samples. Based on 1D and TOCSY spectra (Fig. 5.2), 38 metabolites could be identified. These comprised several amino acids (e.g. leucine, isoleucine, tyrosine, valine), organic acids (e.g. fumarate, malonate), betaine, choline-containing metabolites, glucose and nucleotide-related compounds (e.g. adenosine, AMP and ADP). Lipids were also detected as indicated by the broad signals assigned to fatty acyl chains. The complete list of compounds identified and their respective ^{1}H chemical shifts are presented in Table 5.1. Twenty four out of the 38 metabolites identified in the present study had not been previously reported for terrestrial isopods (marked with an asterisk in Table 5.1).

Table 5.1 List of 500 MHz ^{1}H NMR resonance assignments of metabolites of tissue extract of the terrestrial isopod species *Porcellionides pruinosus*. Only δ^{1}H chemical shifts observed for each metabolite are presented in the list. *- denotes metabolites first time identified in this study. ǂ - denotes the H used for integration purposes. Lipids present the signal are used for integration

Metabolite	δ1H (assignment)
2-Oxoglutarate*	2.45(β-CH2); 3.01(γ-CH2)
Adenosine*	**6.09(C1'-H, ribose)ǂ**; 8.23(C8, ring); 8.34(C2, ring)
ADP*	6.16(C1', ribose); 8.28(C8, ring); **8.60(C2, ring)ǂ**
Alanine*	1.47(β-CH3); 3.77(α-CH)
AMP*	4.01(C5'-H, ribose); 4.35(C4'-H, ribose); 4.46(C3'-H, ribose); 4.77(C2'-H, ribose); 6.04(C1'-H, ribose); **8.57(C2, ring)ǂ**
Arginine*	**1.67(γ-CH2)ǂ**; 1.93(β-CH2); 3.23(δ-CH2); 3.79(α-CH)
Asparagine*	2.87(β-CH); 2.95(β'-CH); 4.01(α-CH)
Betaine	3.27(N-(CH3)3); **3.90(CH2)ǂ**
Choline	**3.21(N-(CH3)3)ǂ**; 3.52(β-CH2); 4.06(α-CH2)
Citrate	2.54(α-, γ-CH); 2.67(α'-, γ'-CH)
Cytidine*	6.06(5H); 7.82(6H)
Fumarate	**6.52(α,β C=C)ǂ**
α-Glucose	3.42(H4); 3.53(H2); 3.70(H3); 3.77(1/26CH); 3.84(1/26CH); **5.25(H1)ǂ**
β-Glucose	3.23(H2); 3.39(H4); 3.42(H3); 3.48(H3); 3.71(H5); 3.89(H6); 4.64(H1)
Glutamate	2.10(β-CH2); 2.36(γ-CH2)
Glutamine	2.15(β-CH2); **2.47(γ-CH2)ǂ**; 3.78(α-CH)
Glycerophosphocholine*	3.68(γ-CH2); 4.32(α'-CH2 P)
Glycine*	**3.56(α-CH)ǂ**
Histidine	**7.08(cyclic 2H)ǂ**; 7.78(C2, ring)
Isoleucine	0.94(δ-CH3); **1.02(γ'-CH)ǂ**; 1.28(γ-CH); 1.48(γ'-CH); 1.99(β-CH); 3.68(α-CH)
Lactate	1.30(β-CH3); 4.13(α-CH)
Leucine*	0.96(δ'-CH3); **0.97(δ-CH3)ǂ**; 1.73(γ-CH); 1.75(β-CH2); 3.73(α-CH)
Lipids CH=CH	5.28-5.34 (m)
Lipids (CH2)n	1.24-1.31 (m)
Lipids CH3	0.84-0.91 (m)
Lysine	1.49(γ-CH2); 1.74(δ-CH2); 1.92(β-CH2); **3.02(ε-CH2)ǂ**
Malonate*	3.11(β'-CH)
Methionine*	2.15(S-CH3); 2.19(β-CH2); 2.65(γ-CH2); 3.87(α-CH)
Phenylalanine	3.15(β'-CH); 3.30(β-CH), 3.98(α-CH); 7.34(cyclic C2, 6); 7.39(cyclic C4); **7.43(cyclic C3, 5)ǂ**
Phosphocholine*	3.22(N-CH3); 3.60(γ-CH2); 4.23(α'-CH2 P)
Proline*	2.01(γ-CH2); 2.08(β'-CH); 2.36(β-CH); 3.35(δ'-CH); 3.42(δ-CH); **4.13(α-CH)ǂ**
Threonine	1.34(γ-CH3); 3.58(α-CH); 4.26(β-CH)

Table 5.1 (cont.) List of 500 MHz 1H NMR resonance assignments of metabolites of tissue extract of the terrestrial isopod species *Porcellionides pruinosus*. Only δ^1H chemical shifts observed for each metabolite are presented in the list. *- denotes metabolites first time identified in this study. ǂ - denotes the H used for integration purposes. Lipids present the signal are used for integration

Metabolite	**δ^1H (assignment)**
Tryptophan	4.06(α-CH); 3.30(β-CH); 3.52(β'-CH); 7.31(C2H, ring); 7.74(C4H, ring); 7.20(C5H, ring); 7.28(C6H, ring); 7.55(C7H, ring)
Tyrosine	3.07(β'-CH); 3.17(β-CH); 3.95(α-CH); 6.90(C3, 5H, ring); **7.20(C2, 6H, ring)ǂ**
UMP*	5.98(C1'-H, ribose); 8.08(C6, ring)
Uracil*	5.81(5H); 7.54 (6H)
Uridine	5.91(Ribose H1); 5.94(cyclic H5); 7.88(cyclic H6)
Valine	1.00(γ-CH3); **1.04(γ'-CH3)ǂ**; 2.28(β-CH); 3.62(α-CH)
Unassigned 1	**1.48 (s)ǂ**
Unassigned 2	**1.50 (s)ǂ**
Unassigned 3	**3.13 (s)ǂ**
Unassigned 4	**5.18 (d)ǂ**
Unassigned 5	**5.20 (d)ǂ**
Unassigned 6	**5.40 (broad)ǂ**

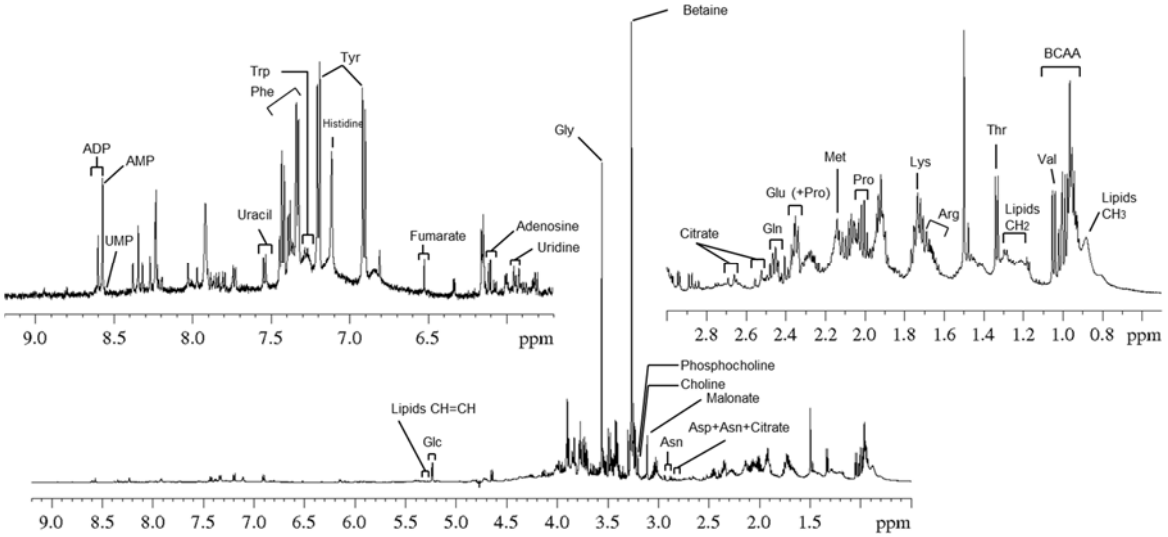

Fig. 5.1 Representative 500 MHz 1D ^{1}H-NMR spectrum of tissue extract of the terrestrial isopod species *Porcellionides pruinosus*. Key: ADP- adenosine diphosphate; AMP- adenosine monophosphate; Arg- arginine; Asn- asparagine; Asp- aspartate; BCAA- branched chain amino acids; Glc- glucose; Gln- glutamine; Glu- glutamate; Gly- glycine; Lys- lysine; Met- methionine; Phe- phenylalanine; Pro- proline; Trp- tryptophan; Tyr- tyrosine; UMP- uridine monophosphate; Val- valine.

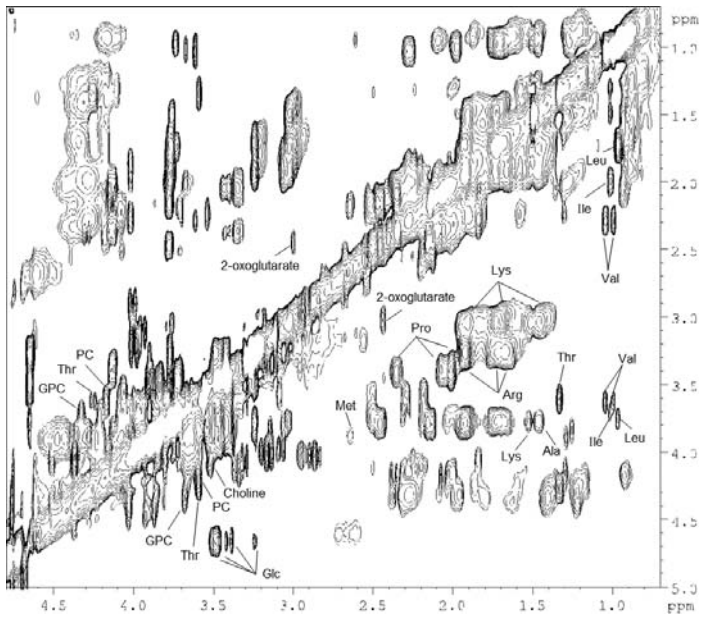

Fig. 5.2 Expansion of a representative 500 MHz ^{1}H-^{1}H TOCSY NMR spectrum of tissue extract of the terrestrial isopod species *Porcellionides pruinosus*. Key: Ala- alanine; Arg- arginine; Glc- glucose; GPC- glycophosphocholine; Ile- isoleucine; Leu- leucine; Lys- lysine; Met- methionine; PC- phosphocholine; Pro- proline; Thr- threonine; Val- valine.

3.2. Metabolic variations in control organisms over time

In order to evaluate the biological variability of the organisms used, the metabolic composition of isopods sampled directly from the laboratory culture (time 0) was compared to that of organisms sampled at subsequent time points in control conditions (4, 7 and 14 days). The corresponding ^{1}H-NMR spectra are shown in Fig. 5.3. A first visual inspection of these spectra suggests that some metabolites vary quantitatively over time, which was indeed confirmed by spectral integration of individual signals, as summarized in the heatmap shown in Fig. 5.4. For each metabolite, this heatmap is colour-coded according to the percentage of variation in control animals, collected at different time points (4, 7 and 14 days), relatively to the pre-exposure time (0 hours). While the levels of some metabolites (e.g. arg, pro, malonate, fumarate, phosphocholine) were found to be stable over time, other compounds showed important changes. Several amino acids (val, leu, ile, lys, gly, glu, gln, tyr, phe), choline and uracil showed a decrease in their levels at day 4 when compared to organisms from time zero (from cultures) and then increased again till the 14th day. With an opposite trend, betaine and glucose increased from time zero to day 4 and then decreased till the end of the test. Finally, another interesting change regarded the levels of AMP and ADP, where AMP significantly decreased from time 0 to day 4 and remained low at subsequent time points, and ADP showed an opposite variation, being significantly increased in isopods sampled at days 4, 7 and 14 relatively to time zero organisms.

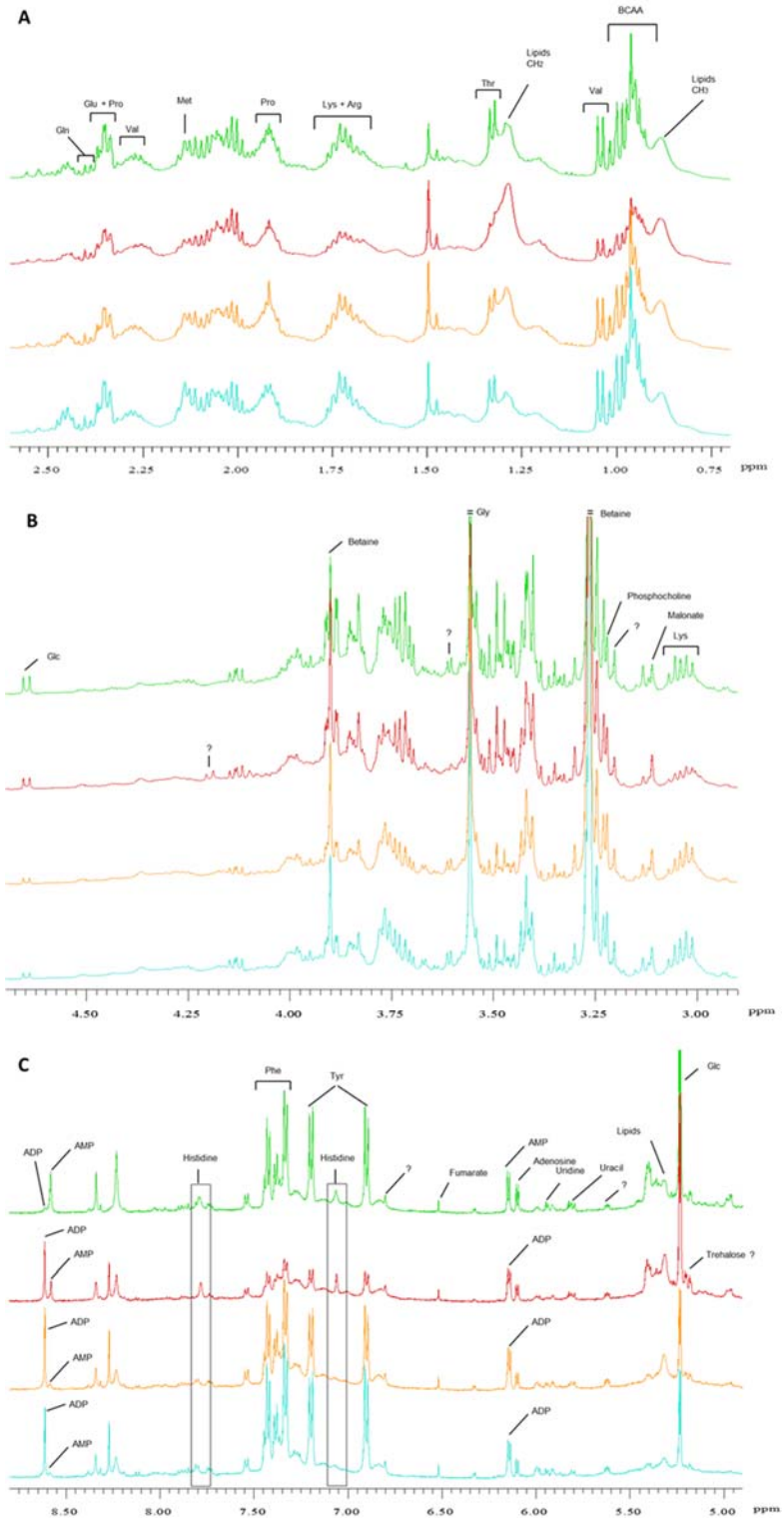

Fig. 5.3 Representative 1D ^{1}H-NMR spectra of tissue extracts from non-exposed organisms (terrestrial isopod species *Porcellionides pruinosus*) before exposure (green spectrum), and at 4 days (red spectrum), 7 days (orange spectrum) and 14 days of exposure (blue spectrum). A) expansion of the aliphatic region; B) expansion of the mid-field region C) expansion of the aromatic region. Key: ADP/AMP- adenosine diphosphate/monophosphate; Arg- arginine; BCAA- branched chain amino acids (isoleucine, leucine and valine); Glc- glucose; Gln- glutamine; Glu- glutamate; Gly- glycine; Lys- lysine; Met- methionine; Phe- phenylalanine; Pro- proline; Thr- threonine; Tyr- tyrosine; Val- valine.

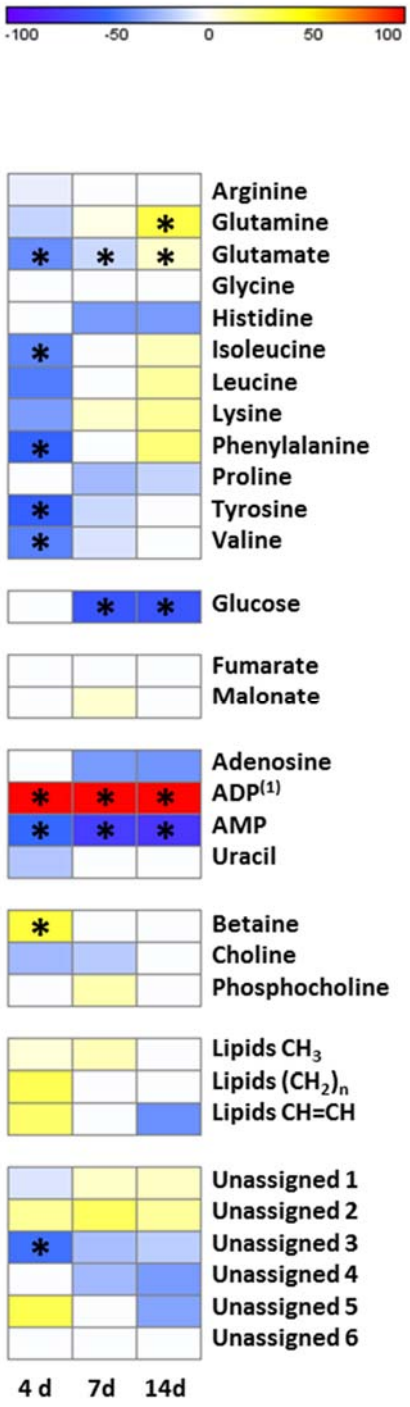

Fig. 5.4 Metabolite heatmap showing the percentage of variation in non-exposed organisms, sampled after 4, 7 and 14 days, relatively to pre-exposure (time 0). Whenever the variation coefficient was higher than the mean variation, no variation was considered. *-denotes significant difference to control (Dunnett's test, α= 0.05 significance); (1) Variation to control was above 100% (respectively 263%, 352% and 281%).

3.3. Nickel-induced metabolic variations

In the nickel exposure trial, several changes in the metabolome of isopods were observed with a dose- and time-dependency, as summarized by the heatmap in Fig. 5.5and shown in more detail in the graphs presented in Fig. 5.6 and Fig. 5.7. Several amino acids (val, ile, leu, lys, gln, glu, tyr, phe - Fig. 5.5) showed an increasing trend relatively to controls at day 4, decreasing afterwards at days 7 and 14. Significant decreases were observed for valine (14 days, both doses), glutamine (14 days, high dose), glutamate (14 days, low dose) and phenylalanine (14 days, low dose). While histidine showed a non-significant decrease at the higher exposure dose, other amino acids did not change upon Ni exposure (arg, pro, gly).

Regarding organic acids (Fig. 5.7), fumarate showed an increment upon Ni exposure after 7 and 14 days, reaching statistical significance in organisms exposed to the high dose. Compared to controls, organisms exposed for 14 days at the high Ni dose had higher levels of malonate although this difference was not significant. Glucose levels were not significantly affected by Ni exposure although there was a trend for a slight increase depicted at 14 days of exposure.

Nucleotides and related compounds, like adenosine, AMP and ADP, seemed to vary in response to Ni exposure, although not reaching statistical significance nor following a consistent pattern (Fig. 5.7).

Betaine was significantly decreased in isopods exposed for 4 days, while at 7 and 14 days the pattern was inverted and levels in exposed organisms were higher than in controls. There was a significant increase in phosphocholine in isopods exposed to the high Ni dose after 7 and 14 days, while choline showed a non-significant trend for increase in exposed organisms at days 4 and 7 (Fig. 5.7).

The amount of total lipids in exposed organisms was significantly altered compared to controls after 4 days of exposure, being decreased at the low dose and increased at the high dose. At 7 and 14 days, there were no significant differences between lipid levels, although with a trend to be decreased in organisms exposed to the high dose, relatively to controls (Fig. 5.7).

Some significant changes were noticed for a few signals not yet assigned. The singlet at 1.48 ppm was significantly increased in isopods exposed for 4 days, whereas the singlet at 3.13 ppm showed a significant increase at 14 days at the high dose of exposure.

Finally, the Two-Way ANOVA analysis showed significant interactions between time and concentration for lipids, valine, glutamine and ADP.

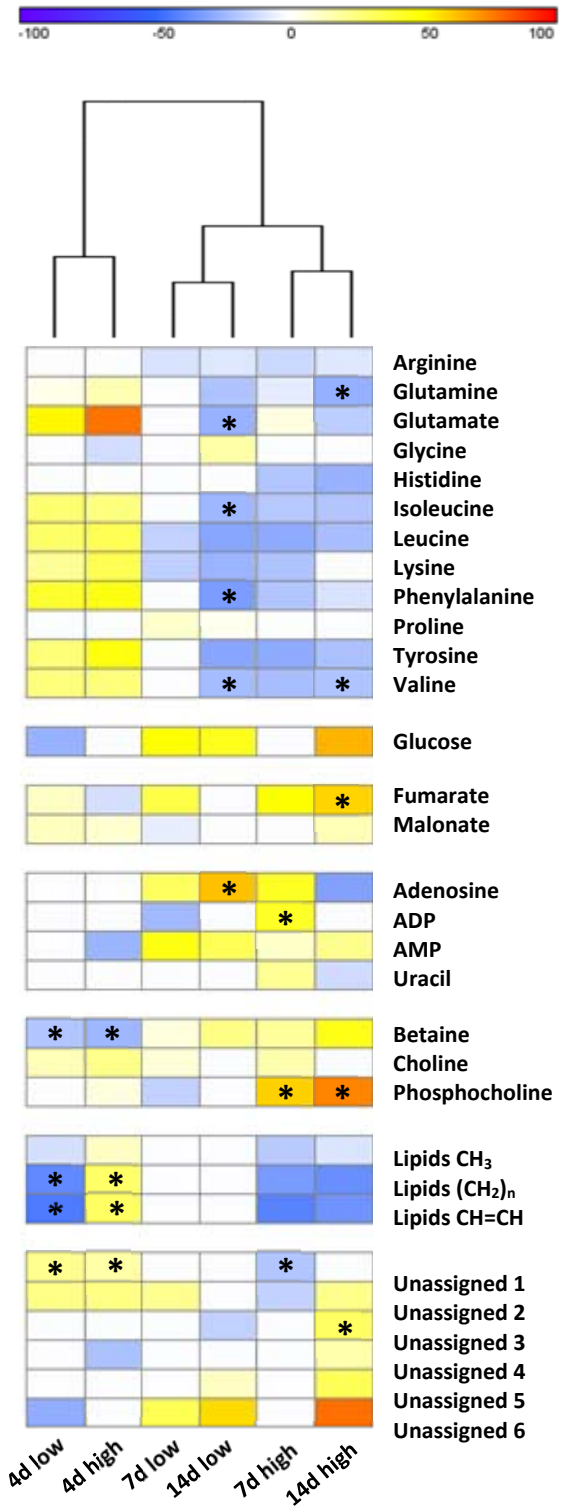

Fig. 5.5 Metabolite heatmap showing the percentage of variation in organisms exposed to two concentrations of nickel (low: 50 mg/kg soil and high: 250 mg/kg soil) and sampled after 4, 7 and 14 days of exposure, relatively to control organisms (collected at the same time points). Whenever the variation coefficient was higher than the mean variation, no variation to control was considered. * - denotes significant difference to control (Dunnett's test, α= 0.05 significance)

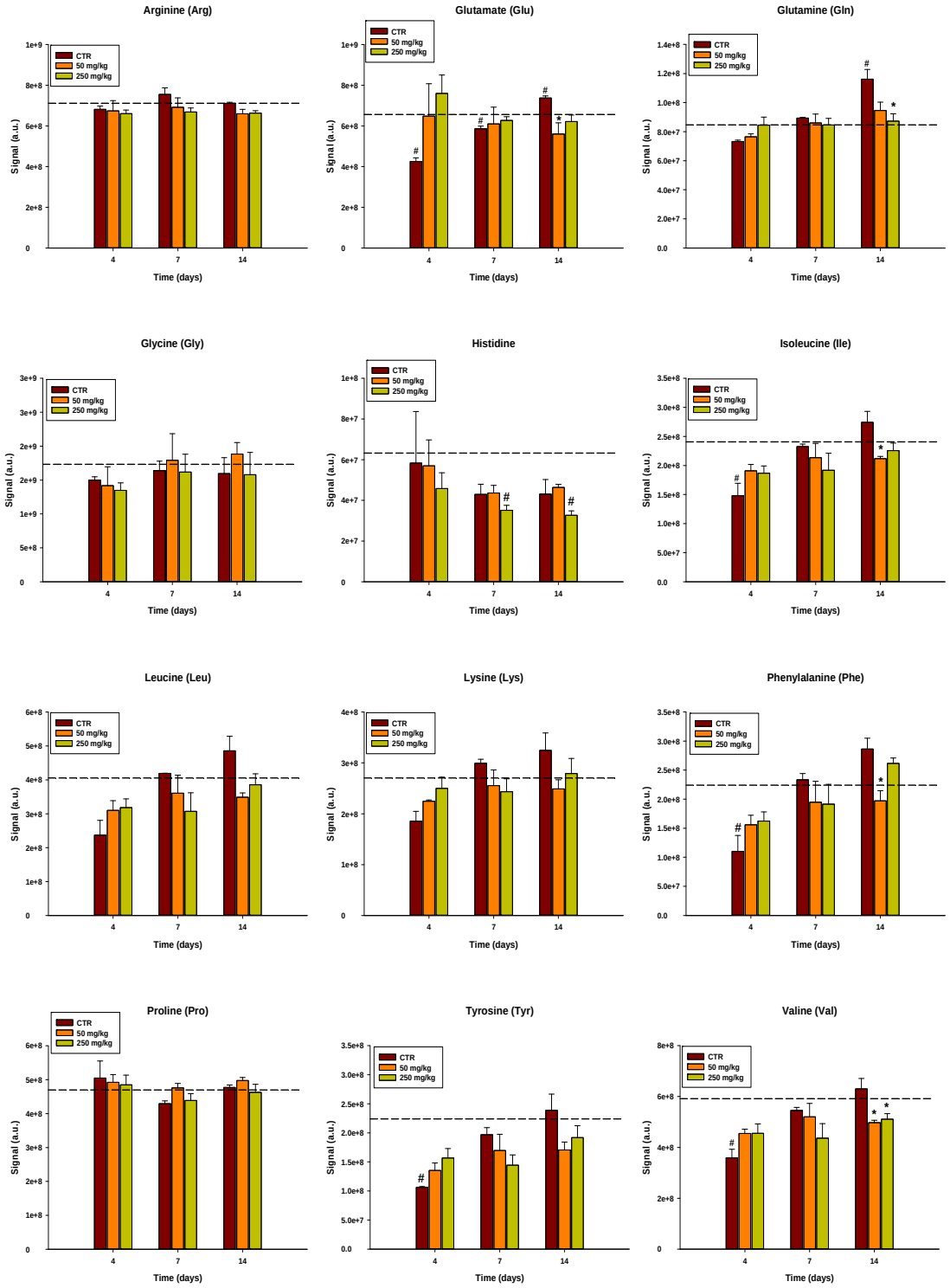

Fig. 5.6 Signal areas (a.u.) of amino acids in organisms sampled at 4, 7 and 14 days of exposure to nickel (50 mg/kg soil and 250 mg/kg soil). The dotted line represents metabolite levels in organisms sampled prior to exposure (time 0); *- denotes statistical difference to control, at the same time point ($p < 0.05$); #- denotes statistical difference to time 0 ($p < 0.05$)

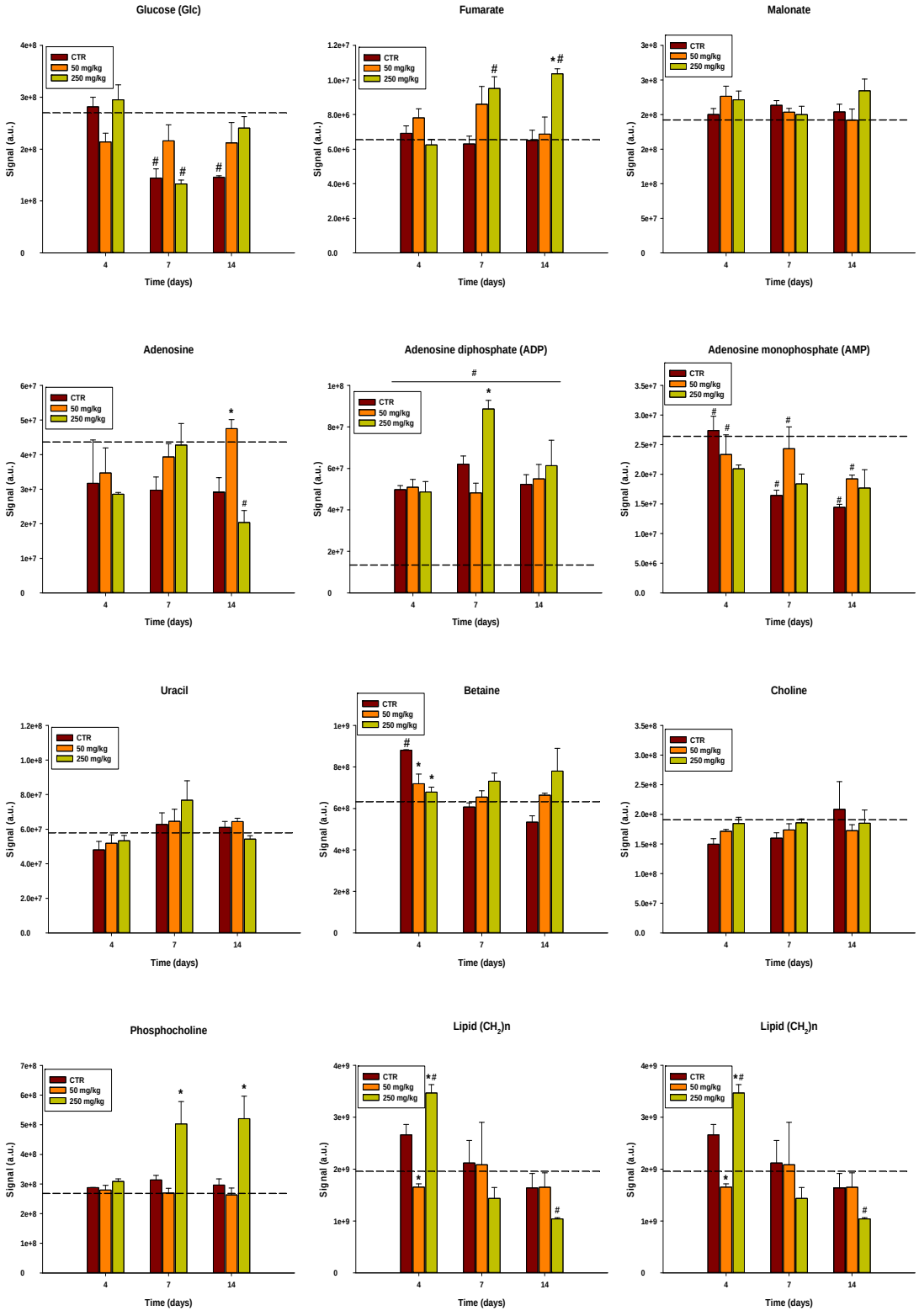

Fig. 5.7 Signal areas (a.u.) of sugars (glucose), organic acids (fumarate and malonate), nucleobases, nucleosides and nucleotides (adenosine, ADP, AMP and uracil), lipids and others (betaine, choline, phosphocholine) in organisms sampled at 4, 7 and 14 days of exposure to nickel (50 mg/kg soil and 250 mg/kg soil). The dotted line represents metabolite levels in organisms sampled prior to exposure (time 0); *- denotes statistical difference to control at the same time point ($p < 0.05$); #- denotes statistical difference to time 0 ($p< 0.05$)

3.4. Dimethoate-induced metabolic variations

A similar analysis was conducted for dimethoate exposure, by measuring the relative variations of metabolites in exposed organisms relative to control organisms, at the three sampling time/points considered (Fig. 5.8 to Fig. 5.10).

Similarly to the pattern already observed for Ni exposure, several amino acids were generally increased relatively to controls at day 4 and then decreased at days 7 and 14. Moreover, arginine which did not present a significant variation upon Ni exposure, was significantly decreased in dimethoate-exposed isopods (day 14, higher dose - Fig. 5.9).

Fumarate showed a trend to increase upon dimethoate exposure after 7 and 14 days, although not significantly. Glucose levels were significantly decreased after 4 days (both doses) but did not vary significantly at the 7^{th} and 14^{th} days. No changes were observed in lipids for organisms exposed to dimethoate when compared to the controls (Fig. 5.10).

AMP and adenosine were higher in organisms exposed to the highest dimethoate dose after 7 and 14 days, respectively. ADP showed a trend to decrease in exposed organisms at days 7 and 14 (Fig. 5.10).

Betaine showed a trend for decreasing after 4 days of exposure, but for the other sampling times levels were similar to controls. Choline compounds did not vary significantly relatively to controls (Fig. 5.10).

Regarding unassigned signals, the singlet at 1.48 ppm was significantly increased in isopods exposed for 4 days at the higher dose, whereas the unassigned broad signal at 5.40 ppm (possibly arising from a sugar moiety) was significantly decreased.

Finally, the Two-Way ANOVA analysis showed significant interactions between time and concentration only for arginine.

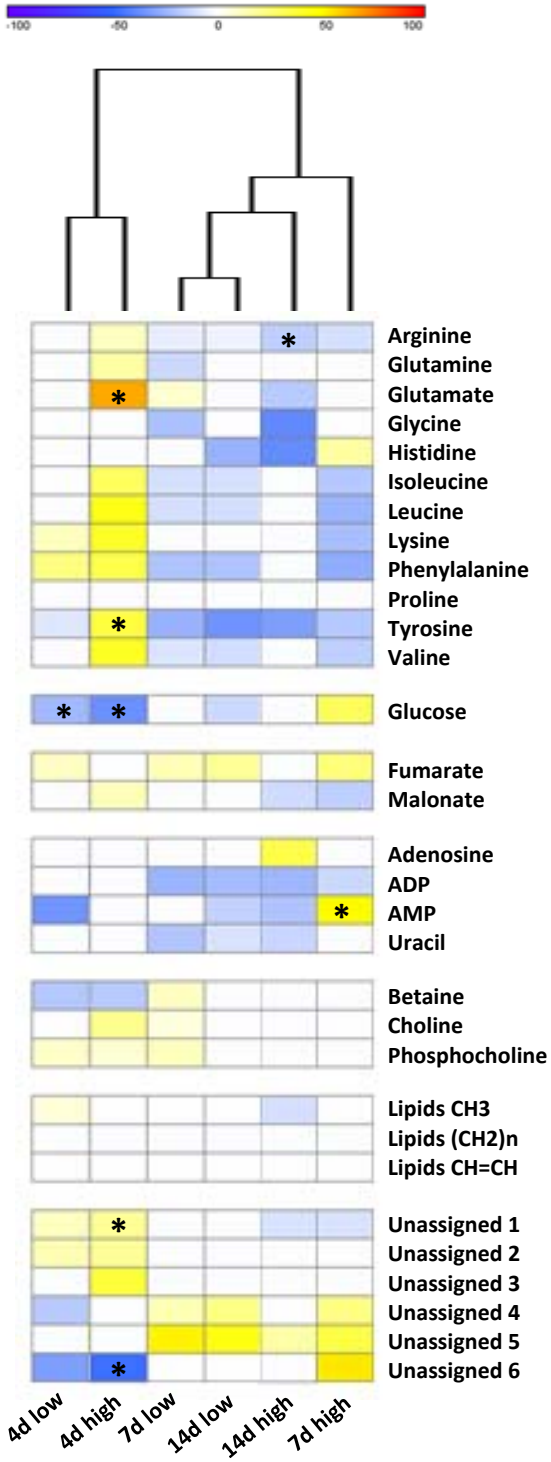

Fig. 5.8 Metabolite heatmap showing the percentage of variation in organisms exposed to two concentrations of dimethoate (low: 0.4 mg/kg soil and high: 10 mg/kg soil) and sampled after 4, 7 and 14 days of exposure, relatively to control organisms (collected at the same time points). Whenever the variation coefficient was higher than the mean variation, no variation to control was considered. * - denotes significant difference to control (Dunnett's test, $\alpha = 0.05$ significance)

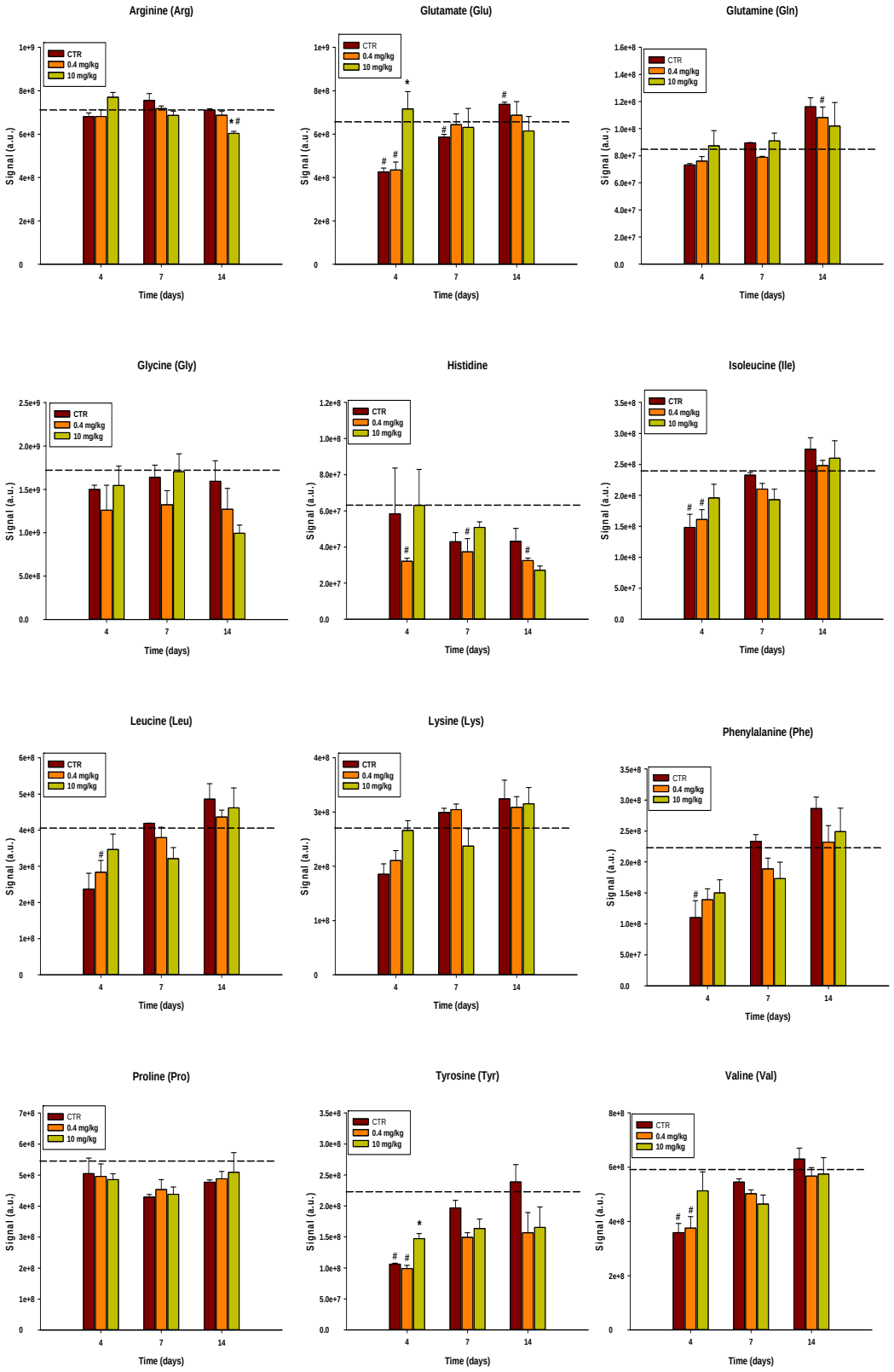

Fig. 5.9 Signal areas (a.u.) of amino acids in organisms sampled at 4, 7 and 14 days of exposure to dimethoate (0.4 mg/kg soil and 10 mg/kg soil). The dotted line represents metabolite levels in organisms sampled prior to exposure (time 0); *- denotes statistical difference to control at the same time point ($p < 0.05$); #- denotes statistical difference to time 0 ($p < 0.05$)

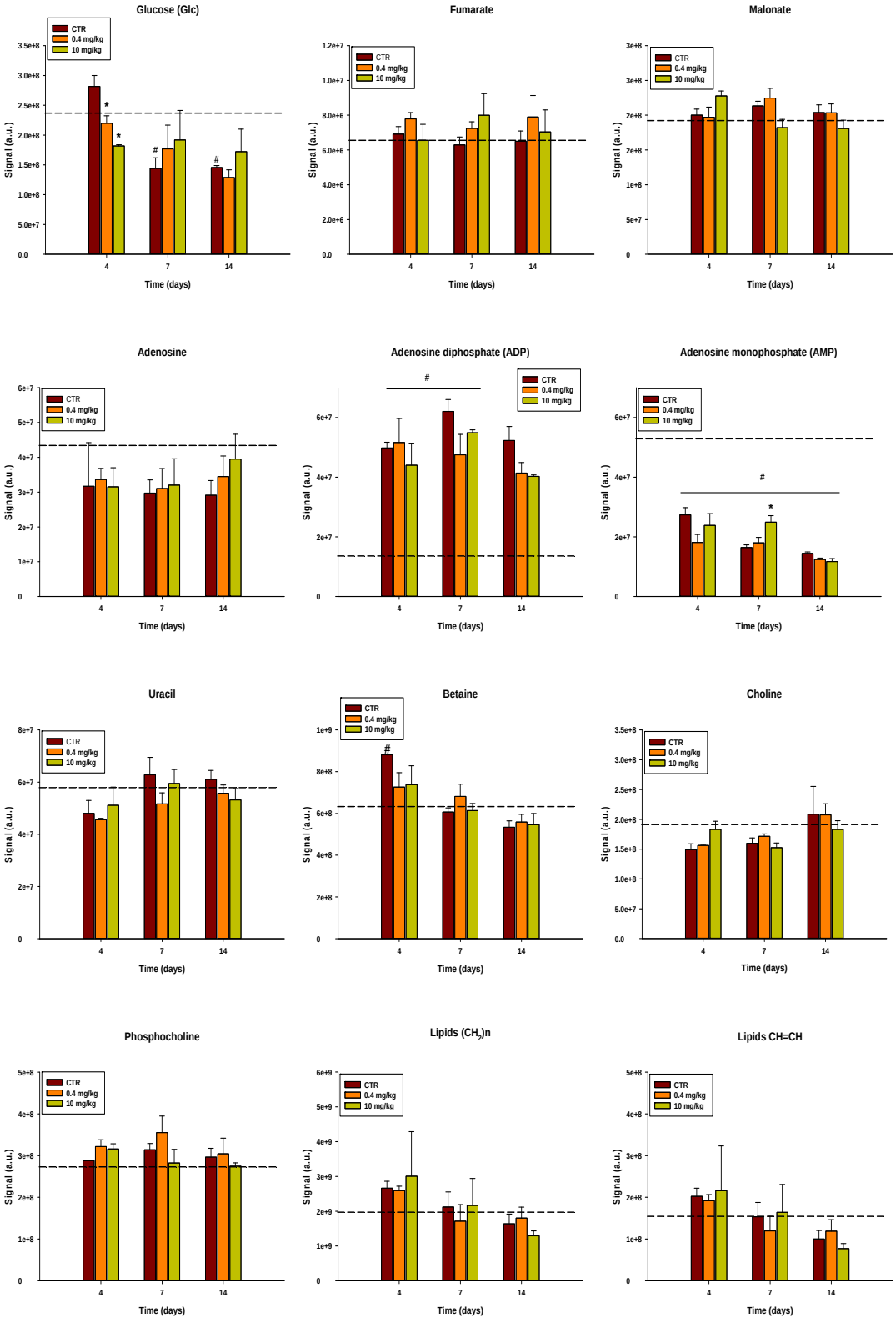

Fig. 5.10 Signal areas (a.u.) of sugars (glucose), organic acids (fumarate and malonate), nucleobases, nucleosides and nucleotides (adenosine, ADP, AMP and uracil), lipids and others (betaine, choline, phosphocholine) in organisms sampled at 4, 7 and 14 days of exposure to dimethoate (0.4 mg/kg soil and 10 mg/kg soil). The dotted line represents the metabolite levels in organisms sampled prior to exposure (time 0); *- denotes statistical difference to control at the same time point ($p < 0.05$); #- denotes statistical difference to time 0 ($p < 0.05$)

4. Discussion

In this work, 1D and 2D NMR methods were used to characterize the metabolic composition of the species *P. pruinosus*. It was possible to identify 24 new metabolites that were not described previously in the work of Gibb et al. (1997) for the species *Oniscus assellus* and *Porcellio scaber.* Some of the newly identified metabolites are considered essential amino acids for crustaceans, such as arginine, histidine, isoleucine, leucine, phenylalanine, tryptophan, valine or methionine (Claybrook, 1983). This higher number of metabolites identified in this work may relate, on one hand, to a more detailed assignment (based on 2D spectra) and, on the other hand, to real metabolic differences between the species. Indeed, this inter-species variability has also been observed in other studies, for instance in the work of Liebeke et al. (2014), where metabolic profiles could be used to distinguish cryptic species of earthworms *Lumbricus rubellus*.

The time course analysis of control organisms provided important information regarding fluctuations in the metabolome of terrestrial isopods. Variations within organisms in control conditions were observed in time, especially between the pre-exposure time (time 0) and day 4. The fact that no distinction between genders was made may possibly account for this variability, as each pool could contain a different ratio of male/female organisms. Another possible explanation is that these variations may result from adaptations in the organisms' behaviour, as terrestrial isopods are considered sociable (Linsenmair, 1984) and even produce chemical clues to interact and aggregate (Broly et al., 2012; Takeda, 1980). These social interactions have been highlighted to affect organisms' fitness according to group size as described by the Allee effect, where it is described that isolated organisms tend to spend more time and energy searching for other individuals with which to aggregate whilst sheltering (Brockett and Hassall, 2005). Therefore, the change from culture boxes where approximately 100 organisms (from mancae to juveniles and adults) co-habit, to boxes with a total of 20 organisms (all adults) will possibly alter the production of such chemical clues and, consequently, the levels in metabolites. When organisms are moved to the test boxes, filled with "new" Lufa 2.2 soil, the structure and the microbiome of the soil is different from the one in the culture boxes. These changes will also possibly affect the metabolite levels as the food available will be differently colonized by microorganisms, which in turn will alter

the availability and acquaintance of essential or non-essential amino acids by the terrestrial isopods. While essential amino acids have to be obtained through diet, the non-essential amino acids are synthetized within organisms (Claybrook, 1983). From the ten amino acids considered non-essential for crustaceans, tyrosine is derived from the essential amino acid phenylalanine, where similar ratios were observed showing therefore a consistent pattern. The other non-essential amino acids are synthetized from glucose, and therefore may explain the decrease on its levels in time under control conditions. Lipids also suffered an initial increase followed by a decrease on the 7^{th} and 14^{th} days, which may also be related to the amino acids' need, as lipidic oxidation will directly affect the Krebs cycle (Porter et al., 1995). As lipids and carbohydrates are being transformed (possibly, to some extent, into amino acids) it was also expected the observed transformation of AMP into ADP, for energy storage. Overall, the above alterations may evidence a strategy for organisms to cope with the change into a new environment, with new physical and chemical parameters, and even with different population densities.

4.1. Exposure to nickel

Metabolic profiling of organisms exposed to nickel showed that different metabolites varied as function of both time and dose. More specifically, by applying hierarchical cluster analysis to metabolite variations, metabolic responses at the first time point (4 days) were found to be independent of the dose of exposure, whereas afterwards (at days 7 and 14) the clustering was based on the concentration of exposure and not on the time of exposure. This result suggests that, at initial exposure times, isopods present high metabolic plasticity to adapt to the presence of metals. Although this was already reported in previous studies (Alikhan and Storch, 1990; Bayley et al., 1997), being related with the organisms capacity to store metals in granules and make them non-bioavailable, this is the first time that this plasticity to handle such different concentrations of metals is reported in terms of the metabolome.

As in other crustaceans, for terrestrial isopods, growth and moult are tightly correlated and dependent on the frequent replacement of their cuticle (Fabritius and Ziegler, 2003).

Although moulting is not considered the main excretion route of metals in these organisms (Hopkin et al., 1985), it has been reported to take part on this process. In a study of Köhler (2002), copper-containing granules were able to dissolve during the moult process allowing their excretion. In addition, moulting cycles can be altered by metal exposure, and the regular 28 days cycle may be changed (Drobne and Štrus, 1996). In the study of Drobne and Štrus (1996), the increase of zinc in contaminated food led to an increase in the number of moults, and in the first week of exposure only approximately 20% of the organisms had moulted in the control, comparatively to approximately 50% at the highest concentration used (10 mg Zn/g food). The metabolites leucine, isoleucine, valine, lysine and phenylalanine (crustaceans' essential amino acids – EAA), tyrosine and glutamine (crustaceans' non-essential amino acids – NEAA, Claybrook, 1983) are known to be involved in moulting related processes (Whiteley and El Haj, 1997). They are involved in several distinctive aspects: the BCAA (branched chain amino acid: leucine, isoleucine and valine) are involved in muscle growth (Shimomura et al., 2006), haemocyanin synthesis and transportation (Bannister et al., 1977); lysine is related to the production of elastin and collagen (Pinnell and Martin, 1968) but also in the absorption of calcium necessary for the formation of the exoskeleton (Fabritius and Ziegler, 2003), phenylalanine and tyrosine in the synthesis of the pigment melanin and haemocyanin transportation processes (Terwilliger, 1999), and glutamine is also involved in muscle growth (Rosa and Nunes, 2005). At both Ni exposure concentrations, the response pattern was similar and involved an increase of these amino acids in the first 4 days of exposure, followed by a consistent decrease to levels lower than the control. These results suggest that exposure of these organisms to nickel may anticipate moulting and some of them may enter the moult process in a period of 7 days. The intensity of the response increased with the increasing of Ni concentrations. This was also observed by (Drobne and Štrus, 1996) in *Porcellio scaber* exposed to zinc-contaminated food, with a trend to moult earlier with an increase in zinc concentrations.

The variations of glutamate and the small variations of glycine observed for the organisms exposed to nickel, may be related to disturbances in the synthesis of glutathione. Glutathione is known to be essential for maintaining the reduction–oxidation (redox) intracellular balance and regulating signalling pathways augmented by oxidative stress (Li et al., 2004). The reduced glutathione form (GSH) is a tripeptide thiol (L-γ-glutamyl-L-cysteinylglycine),

synthetized from two sequential reactions that require the metabolites L-glutamate and L-cysteine in order to form γ-glutamylcysteine, followed by the addition of the metabolite glycine to the C-terminal (Griffith, 1999; Noctor et al., 1998). This possible impact in glutathione synthesis may come in accordance with the activity of glutathione *S*-transferases (GST) and glutathione peroxidase (GPx) observed in the study of Chapter IV. In fact the formation of GSH is used for the formation of GST enzymes, needed to handle reactive oxygen species (ROS), responsible for the induction of DNA damage and impairment of DNA repair (Lynn et al., 1997). Another possible explanation for the levels of glutamate and glycine observed may be related to an impact in GABBA receptors involved in neurotransmission which is more detailed described in the next section. Nevertheless the level patterns observed in the exposures to nickel and dimethoate are different which may indicate the involvement of these metabolites in common, but also in different pathways for metal and pesticide exposures.

In addition, osmoregulation is another mechanism with a crucial role in terms of evolution, for the colonisation of the terrestrial environment by isopods. Organic osmolytes, such as betaine, are small molecules responsible for the maintenance of osmotic balance (Yancey, 2005). This metabolite has also appeared with high levels in other species of isopods (Gibb et al., 1997), and in the present study, although an initial significant decrease is observed for betaine at 4 days of exposure, it shows an increase with time for both Ni concentrations, thus suggesting the disturbance of the osmoregulation process. Fumarate, which may also be associated with osmoregulation and is part of the purine nucleotide and the ornithine-urea cycles involved in nitrogen excretion, was also found to vary.

At 4 days of exposure, the variation in lipids is highly dependent on dose, being decreased for the low dose and increased for the high dose, when compared to controls. However, this increase is no longer observed at longer exposure times, where lipid levels are either similar to controls (low dose) or smaller (high dose). Although the role of these variations is not clear, it may relate to the involvement of the hepatopancreas in the detoxification process. The hepatopancreas '*B*' cells suffer a 24 hour period cycle, during which the cytoplasm is recharged with lipids, glycogen and Fe rich inclusions that are then discharged in order to expel the metals accumulated (Hames and Hopkin, 1991).

The metabolites choline and phosphocholine play important functions in metabolism. Choline is an essential constituent of phospholipids, present in membranes, but also of acetylcholine, a neurotransmission mediator (Simon, 1999). Through the action of choline kinase, choline will be phosphorylation into phosphocholine which will latter be transformed into phosphatidycholine (Zeisel, 1990). Besides phosphatidycholine, choline is also a constituent of other phospholipids such as lysophosphatidycholine, sphingomyelin, lysophyngomyelin and plasmalogens (Simon, 1999). The function of these phospholipids include the maintenance of the integrity and function of organelle and cell membranes (Simon, 1999). But choline can also be acetylated by the enzyme choline acetyltransferase in the presence of acetyl-CoA to form acetylcholine (Simon, 1999), a neurotransmitter molecule that is degraded by acetylcholinesterase (AChE) in the synaptic cleft (Soreq and Seidman, 2001). Although a clear separation between the previous processes (and other processes in which choline is involved such as its irreversible oxidation to betaine - Zeisel, 1990), some considerations can be taken by comparison to previous studies. In Chapter IV, organisms from the same species (*P. pruinosus*) where exposed to identical concentrations of Ni, the enzymatic activity of AChE and the lipid peroxidation rate (LPO) were measured. The LPO showed no significant differences and AChE activity was significantly higher after 14 days of exposure. Although caution should be taken when comparing our study to the study of Chapter IV, results may indicate that choline is being phosphorylated into phosphocholine, which can then be transformed into other phospholipids. These new phospholipids can then be used to replace the peroxidised ones in the membrane thus maintaining the LPO rates similar to control. Also, since the transformation of phosphocholine into cytidine diphosphocholine (an intermediary step in the pathway to the transformation into phosphatidycholine) is rate limiting, the acetylcholine transformation pathway may be activated (Simon, 1999; Zeisel, 1990). This activation would allow the maintenance of low choline levels and would also explain the higher levels of the enzyme AChE in the organism. An increase in AChE levels would be necessary in order to hydrolyse acetylcholine and thus prevent the over-accumulation of this neurotransmitter that consequently, would lead to an overstimulation of cholinergic receptors and the disruption of the nervous system function (Mileson et al., 1998).

Other metabolites apparently affected by Ni exposure, although not at statistically significant levels, were glucose (which is transformed to chitin and therefore involved in moulting), fumarate (a key intermediate in Krebs cycle), malonate (an inhibitor of oxidation in Krebs cycle - Pardee and Potter, 1949), AMP and ADP, therefore suggesting a modest impact on energy metabolism.

4.2. Exposure to dimethoate

The impact of dimethoate on the isopods' metabolome was found to depend on concentration and time of exposure. In particular, the metabolic responses resulting from short-term exposure (4 days) to low and high doses clustered together, while for prolonged exposure (7 and 14 days), clustering occurred as a function of dose.

Similarly to the results obtained for Ni exposure, growth, moult, haemocyanin formation and transport were suggested to be impaired for organisms exposed to dimethoate. Indeed, the metabolites lysine, valine, leucine, isoleucine, arginine, phenylalanine and tyrosine followed a similar pattern. This general response was observed presented in the study of Ferreira et al. (2015) where the same species (*P. pruinosus*) exposed also to dimethoate showed that organisms tend moult in order to cope with dimethoate's stress. Nevertheless, one should be cautious regarding the direct association between growth rate and moulting process as they may not be interconnected. Although more moulting events could occur in exposed organisms throughout their life, this does not mean that those organisms may achieve bigger sizes than non-exposed organisms (with less moulting events). This phenomenon has been previously observed in a study with collembolans and it is linked with the release of xenobiotics (Folker-Hansen et al., 1996).

Significant changes in glutamate levels and even the non-significant changes of glutamine and choline levels after dimethoate exposure may be possibly indicative of neurotoxicity. The first two metabolites (glutamate and glutamine) may be inter-converted in the central nervous system (CNS - Newsholme et al., 2003). In this process, glutamine is released from astrocytes, transported into neurons and converted to glutamate (Behar and Rothman, 2001),

and its accumulation can be considered the cause for neuronal overactivation (Gupta et al., 1984). In a similar way, the metabolite choline is cleaved from acetylcholine by acetylcholinesterase (AChE), and the inhibition of this enzyme activity leads also to a neuronal overactivitation (Soreq and Seidman, 2001). Also in the study of Gupta et al. (1984), a correlation between the three metabolites in different muscle cells, AChE and GABBA receptors are presented. The variations observed for these metabolites in the present study are concordant with the AChE analysis performed in a previous study, where at the lowest dimethoate concentration, almost no AChE inhibition was observed, whereas at the higher concentration high inhibitions were observed (Chapter III). In the study of Yuk et al. (2013), earthworms exposed to the pesticide endosulfan also showed an impairment in the glutamine/GABBA-glutamate cycle. Although endosulfan acts as endocrine disruptor, changes in levels of these two metabolites are similar to the ones observed in our study.

The results also suggest disturbances in the energetic metabolic pathways. In the case of glucose, a decrease of approximately 20 to 30% after 4 days of exposure may be indicative of disturbances in energy metabolism or be associated with efforts/costs in the detoxification processes. The metabolites ADP and AMP, associated with energy transfer in cells, and the metabolites fumarate and malonate, associated with the Krebs cycle, followed similar patterns to the ones observed for the nickel exposure (over time fumarate tends to increase, ADP tend to decrease and AMP shows a significant increase after 7 days of exposure and decreases after 14 days). Identical impairments on energy metabolism due to dimethoate exposure have been also reported in previous studies, where glycogen and pyruvate levels decreased in the catfish *Clarias batrachus* (Begum and Vijayaraghavan, 1995) and in the snail *Lymnaea acuminate* (Tripathi and Singh, 2002).

The organic osmolytes betaine and glycine, involved in osmoregulation, showed an increase upon dimethoate exposure, which is similar to the study of Yuk et al. (2013) where earthworms from the species *Eisenia fetida* were exposed to endosulfan, and these metabolites' levels were related to osmoregulation. Nevertheless, glycine levels should also be analysed carefully, as this metabolite has important roles in other pathways, such as neurotransmission or glutathione biosynthesis. As reported in the study of Vale et al. (2003), a decrease in glycine levels was observed when cultured cerebellar granule cells were exposed to the pesticide lindane. Identically, the possible impact in glutathione biosynthesis

might occur as reported in a previous study (Ferreira et al., 2015), with alterations in GST and GPx activities.

5. Conclusions

The approach presented in this study is a first step to establish a metabolic profile for the isopod species *P. pruinosus* and to understand responses at the individual and enzymatic level, complementary to what has been described in other studies presented in this thesis. Twenty four metabolites were reported for this species for the first time, which include essential and non-essential amino acids, organic acids, nucleobases, nucleosides and nucleotides and other metabolites.

Metabolic profiling of organisms exposed to nickel suggested impairments on growth, moult and haemocyanin synthesis, along with glutathione biosynthesis inhibition, energy metabolic pathways and osmoregulation disturbances. Moreover, short-term exposure to nickel caused significant changes in lipids, possibly in relation to the role of hepatopancreas '*B*' cells daily cycle in metal detoxification processes.

The metabolic profile of organisms was also changed upon dimethoate exposure, with variations possibly reflecting, again, moulting-related changes, disturbances in osmoregulation, energy metabolism and, additionally, disturbances on neurotransmission. Interestingly, the impact on lipids and choline-containing compounds was much smaller than in the case of Ni exposure thus showing changes in the mode of action of each stressor

Results from this study can be used as a foundation to develop other studies on the toxicity effects of xenobiotics to terrestrial isopods, and can assist the more detailed interpretation of processes already described in regular ecotoxicity studies or reported changes in enzymatic biomarkers and energy reserves.

Acknowledgment

The authors would like to thank the laboratorial support given by Dr. Abel Ferreira. The Portuguese National NMR (PTNMR) Network, supported with FCT funds, and the Associated Laboratory CICECO (project FCOMP-01-0124-FEDER-037271, ref. FCT PEst-C/CTM/LA0011/2013) are also acknowledged.

Financial Disclosure

This study was supported by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia within the research project CLIMAFUN- CLImate Changes and Potencial Impact on Soil FUNctional Ecology (FCOMP-01-0124-FEDER-008656), the project PEst-C/MAR/LA0017/2013, and through a PhD grant (SFRH/BD/65739/2009) attributed to Nuno G. C. Ferreira. The funding institutions had no role on the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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CHAPTER VI

Tracking of novel potential biomarkers in Porcellionides pruinosus (Isopoda) exposed to nickel: a transcriptomic approach.

Tracking of novel potential biomarkers in *Porcellionides pruinosus* (Isopoda) exposed to nickel: a transcriptomic approach.

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Abstract:

The first full body transcriptome of a terrestrial isopod is presented in this study, along with its homologies to all the Metazoan phyla and orders, and class within the Arthropoda phylum. This study also presents a Gene Ontology (GO) classification of the obtained transcripts along with clusters of orthologous groups (COG) and a metabolic pathways description by KEGG analysis.

For that, terrestrial isopods from the species *Porcellionides pruinosus* were used as model isopod species whose main chemical exposure route is soil. This species has been often used to assess xenobiotics' toxicity in soils, accounting for their important function as detritivorous in edaphic systems.

The final part of this study presents an RNA-Seq analysis of gene regulation for the exposure to Ni and a GO enrichment analysis. For that *P. pruinosus* was exposed for 96 h to two concentrations of the metal nickel: maximum concentration allowed in the Canadian framework guideline (50 mg Ni/kg soil) and 5x the concentration (250 mg Ni /kg soil). Results showed an impact at genetic and epigenetic levels, in ion trafficking and storage, the generation of oxidative stress, neurotoxicity and reproduction impairment.

***Keywords*:** transcriptome, RNA-Seq analysis, terrestrial isopods, metal trafficking.

1. Introduction

Arthropods are a major phylum with the most morphologically diverse organisms (Zeng et al. 2011), but despite its diversity some orders still present very scarce genomic information. With the recent advances in massively parallel cDNA sequencing (RNA-Seq) that reduced the costs and increase the quality and amount of data acquired, genome and transcriptome sequencing became more cost-effective and present themselves as perfect tools for filling the lack of information for many groups of organisms (Grabherr et al., 2011; Simpson et al., 2009). The transcriptome includes the total complement of messenger RNA (mRNA) produced in a specific cell, tissue or organism and include higher complexity than the genome itself since it presents for example alternative splicing variants (Szabo 2014). In ecotoxicology the use of transcriptomic and gene expression analysis brings essential information necessary to complement the traditional assays (e.g. reproduction or mortality) thus providing new information to legislators and environmental regulators. In fact, even in exposure scenarios were low toxicity is observed alterations may always occur at the transcriptional level (Gibb et al. 2011; Szabo 2014).

The order Isopoda and most specifically terrestrial isopods, lack a proper genome or even transcriptome sequence to serve as base for molecular analysis. In fact, an analysis through databases show that this group has a low number of transcripts that is not related to barcoding mainly due to a transcriptome study by Chevalier et al. (2012) using the species *Armadillidium vulgare* but performed only in the reproductive system of females infected and non-infected with a Wolbachia strain. Isopods present several features that make them a very interesting organism to study and understand in terms of transcriptome. Isopods have been adopted as an ecotoxicological species because they are considered sensitive organisms to organic compounds (e.g. pesticides) but on the other hand they have a high capacity to handle metals ions at high concentration levels (Ribeiro et al., 1999). They have a 4-tube organ, the hepatopancreas, comprised of two very different metal-sequestering cell types handling Cu and Fe, respectively. One type ('*S*' cell) is small and cone shaped, containing cuprosomes (Cu and S-rich organelles) that participate in haemocyanin synthesis. The other cell type ('*B*' cell) is larger, with Fe (and PO_4-rich) inclusions. 'S' cell residence time is long and '*B*' cells discharge their lipid and Fe contents diurnally in a feeding cycle (Hames and Hopkin 1989). For our study, the terrestrial isopod *Porcellionides pruinosus* (Brandt 1983)

was chosen due to their role within the food-chains, and as macrodecomposers, intervenient in nutrient recycling or structure maintenance (Ferreira et al. 2010; Loureiro et al. 2006; Zimmer 2002; Zimmer et al. 2003).

Due to the specific characteristics of terrestrial isopods it was decided to use the model metal nickel. Nickel (Ni) is a trace elemental metal that although it is naturally occurring, high input into aquatic and terrestrial ecosystems may occur from anthropogenic activities, such as mining, smelting, coal-gas processing, steelworks, foundries, transport and storage yards or even waste incineration activities. In Europe the production of Ni in the year 2000 was around 182,000 tonnes, representing 20% of the worldwide total (Environmental-Agency 2009). This anthropogenic activity and particularly the application of wastes to land, such as sewage sludge, results in occurrence of localised areas highly contaminated with this metal (Alloway and Alloway 1995). Nickel is known to be a micro nutrient for organisms however, natural occurring deficiency is rare (Cartañá et al. 1991; Schroeder et al. 1974). Although Ni is a known cofactor in marine algal species and plants being exploited within the urease enzyme (Rees and Bekheet 1982; Welch 1981), information regarding its biochemical role in the animals is sparse. Also, the limited data for invertebrate organisms is primarily focused on molecular studies on aquatic organisms (e.g. Vandenbrouck et al. 2009). Nickel is considered a carcinogenic metal and has been proven to impact the transcription of genes related to haemoglobin, transcriptional and translational processes and even the phosphate cycle (Lee et al. 1995a; Pane et al. 2003; Vandenbrouck et al. 2009).

Therefore, the major aims of this research was: i) to present the first global descriptive transcriptome for terrestrial isopods, gene annotation and pathway mapping, using the species *Porcellionides pruinosus*; ii) to perform a gene comparison with other close related organism; iii) and to analyse the global transcriptome of isopods' exposure to nickel.

The results obtained will serve as an "open door" for the development of new research lines on the molecular biology of terrestrial isopods, and will also bring new insights to the actual knowledge of nickel and its effects on terrestrial organisms.

2. Materials and methods

2.1. Test Organism and Culture Procedure

The organisms used in this study belong to the species *Porcellionides pruinosus* Brandt (1833), and were previously collected from a horse manure heap and maintained for several generations in laboratory cultures. In culture, isopods were fed *ad libitum* with alder leaves (*Alnus glutinosa*) and maintained at 22 ± 1°C, with a 16:8 h (light:dark) photoperiod. Twice a week cultures were water sprayed and food provided whenever necessary.

2.2. Soil Spiking

LUFA 2.2 soil was spiked with nickel (II) sulfate hexahydrate ($NiSO_4.6H_2O$) with two concentrations: 50mg and 250 mg nickel/kg soil, with a final moisture content equivalent to 50% of the soil water holding capacity. The concentration of 50 mg nickel/kg soil represents the maximum concentration allowed by the Canadian framework guideline (CBP, 2010) and the concentration 250mg nickel/kg soil, represents 5x this maximum concentration. Both concentrations can be found in the field in European, and also in Portugal (Figueira et al., 2002).

2.3. Experimental procedure: transcriptome analysis

Toxicity tests were performed in plastic boxes (14 length x 9 width x 5 height cm), containing approx. 2cm height of natural LUFA 2.2 soil layer (Speyer, Germany) and 5 isopods (per box). Test organisms were collect from culture boxes, weighted (15 - 25mg) and placed in each test-box. No distinction between genders was made, but pregnant females were excluded from trial. Animals with abnormalities and apparent moulting were also excluded. Alder leaf disks (Ø 10 mm, ± 20 mg) were supplied as food, but using a quantity that prevented organisms to remain on top, avoiding contaminated soil.

Organisms were exposed for a 96 h period with 16:8h (light:dark) photoperiod, at 20ºC ± 1°C, after which they were collected, transferred to Trizol ® and stored at -20ºC for total RNA extraction, for a period not longer than a week.

2.4. RNA extraction

Total RNA was extracted from each individual organism and using Trizol® Reagent (Ambion, UK) followed by a column purification step using RNAeasy Mini Kit® (Qiagen, UK) and stored at -80°C. Prior to freezing, analysis on the A260/280 and A260/230 ratios for all RNAs prepared was measured using Nanodrop 2000c spectrophotometer (Nanodrop Technologies, USA).

2.5. Library constructions and sequencing

A total of four expressed sequence tags (EST) libraries were prepared from full organisms of *P. pruinosus*. A pair-end library was performed by mixing equal amounts of total RNA extracted separately from organisms from different life stages: mancae, juveniles and adults (males and pregnant females). Three single end libraries were also performed corresponding to organisms in control treatments and exposed to nickel (50 mg and 250 mg/kg soil). Each of the single end library was performed by mixing equal amounts of total RNA extracted separately from three of the five exposed organisms that had the best extractions. The samples were then sent to Baseclear (BioSciencePark of Leiden, The Nertherlands) that was responsible for the preparation of the libraries and their sequencing. In a very simple description the mRNA was purified by polyA capture, fragmented, converted to double-stranded cDNA. The fragments used for sequencing had an average length in the range of 100-200 bp.

Fragments were sequenced in an Illumina Hi-Seq platform and provided already trimmed for the Illumina adaptors from Baseclear (BioSciencePark of Leiden, The Nertherlands).

Sequencing generate approximately 17.5 Million 50bp sequences for each sample and a quality check was performed using FastQC (v.0.10.1 Babraham Bioinformatics), and presented scores above 32 for all samples.

2.6. EST assembly and redundancy assessment

The transcriptome was assembled according to the procedures described by Jain et al. (2013) and Zeng et al. (2011). For the assembly the pipelines used were: Velvet/Oase with *k*-mers ranging from 21-39 nt (Schulz et al., 2012; Zerbino and Birney, 2008), Trans-ABySS, with *k*-mers ranging from 19-35 nt (Simpson et al., 2009), SOAPdenovo-Trans, with *k*-mers ranging from 17-33 nt (Xie et al., 2013), Trinity (Grabherr et al., 2011) and CLC Genomics® (default parameters were used for all the assemblies).

All the assemblies were used within the EvidentialGene Software package (http://arthropods.eugenes.org/EvidentialGene/), from where resulted the final assembly defined as the Model Assembly (MA).

2.7. Homology search and functional annotation

Homologies search were carried out by query of the National Center for Biotechnology Information (NCBI) non-redundant (nr) database using the Blastx algorithm (v.2.7.0 E-value cut-off of $1e^{-5}$ - Altschul et al. 1997). Afterwards it was used with Blast2GO program (v.2.7.0 - Conesa et al. 2005) to obtain the Gene Ontology (GO) annotation, Enzyme Commission number (EC) terms and biochemical pathway information from the KEGG database (Kanehisa and Goto, 2000). The same procedure was performed individually against the Metazoa and Viridiplantae kingdom, the phyla: Porifera, Cnidaria, Ctenophora, Nematoda, Arthropoda, Platyhelminthes, Annelida, Mollusca, Echinodermata and Chordata. Within the phylum Arthropoda annotations were performed against the subphyla Chelicerata, Crustacea, Hexapoda and Myriapoda and the orders from the subphylum Crustacea:

Amphipoda, Cumacea, Mysidacea, Tanaidacea and Isopoda (order in which the studied species is included); the orders Mictacea, Spelaeogriphacea and Thermosbaenacea were not included since no protein sequences were found. The classification presented before was based in ITIS (Integrated Taxonomic Information System - http://www.itis.gov/).

2.8. RNA-SEQ analysis

RNA-Seq analysis was performed by mapping the control and treatments against the MA, followed by a counting, normalisation and statistical identification of different fold regulation for treatments when compared between each other and the control. The cut-off values to determine different expressed sequences was based in the study of Dalman et al. (2012). The authors showed that the use of arbitrary fold change cut-offs > 2 can provide important information that cannot be obtained only when working with *p* values. Since our study presents for each treatment a pool of organisms the use of cut-offs will be appropriated for the RNA-Seq analysis. Another RNA-Seq analysis was performed as described by Anders et al. (2013). A second analysis was performed in the basis that the mean is a good predictor for dispersion, so *"two samples from different conditions and a number of genes with comparable expression levels, of which we expect only a minority to be influenced by the condition, we may take the dispersion estimated from comparing their counts across conditions as ersatz for a proper estimate of the variance across replicates"* – DESeq package (Anders and Huber, 2012). This second analysis used the same mapping described for the first analysis and is presented in the Supplementary Data. Afterwards an enrichment GO analysis was performed using DAVID software (Huang et al., 2008) and networks were generated in REVIGO software (Supek et al., 2011) using the data provide from DAVID software (Cytoscape® was also used in order to prepare the network presented).

3. Results

No mortality was observed for the 96 h exposures of *P. pruinosus* to Ni.

3.1. Transcriptome sequencing output and assembly

The Illumina high-through-put sequencing produced 88,315,479 reads each containing 51nt, representing approx. 4.50 Gb nucleotides. The result of the EvidencialGene software is presented on Table 6.1. In a short description a total of 535,772 input transcripts, gave 61.5% redundant identic and 38.5% different ones. The final sequence file had a total of 41,771 transcripts (24%) in 21,053 loci which were used as the MA (**Erro! A origem da referência não foi encontrada.**). From the initial 173,843 transcripts 75.9% (132,072) were discarded, and 11.9% (20,718 transcripts) were considered isoforms of the ones used in the MA.

Table 6.1 EvidencialGene software output data for *Porcellionides pruinosus*. Data are presented as percentage (number of transcripts). Transcript class description is: hi – high identity (≥ 98% DNA); hi1 - (very hi identity) and a2 (protein identity) are subclasses; mid and mfrag are lower identity subclasses.

Class	Okay	Discarded
Alternate transcript with main identified (hi)	35% (6135)	6.1% (10635)
Alternate transcript with main identified (hi1)	6.4% (11293)	26.7% (46430)
Alternate transcript with main identified (hi a2)	0% (0)	0.5% (993)
Alternate transcript with main identified (mfrag)	0.4% (780)	0.4% (838)
Alternate transcript with main identified (mfrag a2)	0.1% (250)	0.1% (236)
Alternate transcript with main identified (mid)	1.1% (1949)	1% (1893)
Alternate transcript with main identified (mid a2)	0.1% (311)	0.1% (188)
Primary transcript with alternates	8.3% (14546)	6.8% (11858)
Primary transcript with alternates (a2)	1.1% (1929)	0.5% (1018)
Primary with no alternates	2.6% (4531)	14.6% (25494)
Primary with no alternates (a2)	0% (47)	0% (75)
Fragment alternates (hi)	0% (0)	7% (12293)
Fragment alternates (hi1)	0% (0)	103% (18050)
Fragment alternates (hi a2)	0% (0)	1.1% (2071)
total	24% (41771)	75.9% (132072)

Table 6.2 Summary of the MA transcriptome for *Porcellionides pruinosus*.

Name	Number
N 50	1,485
GC content	39.30%
Minimum transcript	123
Maximum transcript	26,850
Average length	1,007
Numer of BLAST hits	12,055
Total number of transcripts	21,053
Total number of nucleotides	21,202,443

3.2. Homology search and functional annotation

The BLAST performed gave a total of 13,828 annotated transcripts (65.68%). The percentage of homologies with each specific clade is presented in Fig. 6.1. For the top BLAST hits, the phylum Ctenophora, the subphylum Myriapoda and the orders Amphipoda, Cumacea, Lophogastrida, Mysidacea, Tanaidacea presented no hits. The descending order of phyla with top BLAST hits were: Chordata (8,501 hits), Arthropoda (2,610 hits), Nematoda (245 hits), Mollusca (30 hits), Echinodermata (30 hits), Cnidaria (13 hits), Annelida (11 hits), Platyhelminthes (3 hits) and Porifera (1 hit). Within the Arthropoda phylum, the Hexapoda subylum had 2,263 hits, followed by Crustacea (250 hits) and Chelicerata (97 hits) subphyla and finally the order Isopoda (order from the Crustacea subphylum – 8 hits). As for the other clades, Protozoa had 183 hits, followed by Fungi (135 hits), Bacteria (116 hits), Plantae (112 hits) and Virus (42 hits).

When analysing the total number of BLAST hits within each specific clade, the Arthropoda phylum had highest hits with 13,520 and was followed by Chordata (12,362 hits), Mollusca (12,161 hits), Echinodermata (11,591 hits), Cnidaria (11,590 hits), Annelida (11,556 hits), Nematoda (10,706 hits), Platyhelminthes (9,316 hits), Porifera (8,879 hits) and Ctenophora (1,478 hits). Within the Arthropoda phylum, the subphylum Hexapoda had the highest hits – 13,145, followed by Crustacea (12,006 hits), Chelicerata (11,691 hits) and Myriapoda (1,425 hits). The order with more hits was the Amphipoda (842 hits), followed by Isopoda (669 hits), Mysidacea (55 hits), Lophogastrida (9 hits) and Tanaidacea (1 hit), and finally the Cumacea order with no hits.

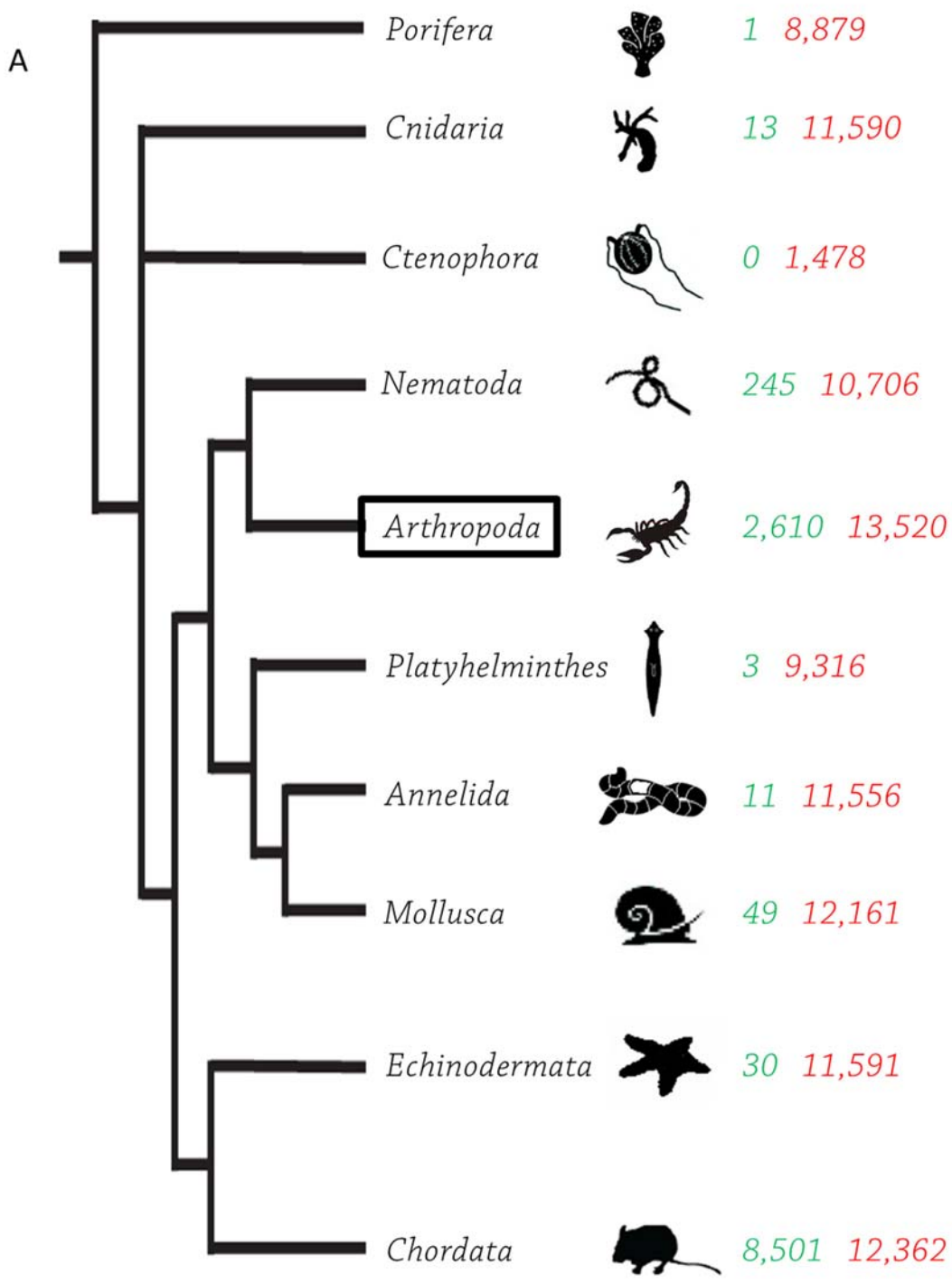

Fig. 6.1 Phylogenetic distribution of species of BLAST hits for the *Porcellionides pruinosus* transcriptome transcripts. A – BLAST hits for Metazoa phyla. B – Total number of BLAST hits for some subphyla and orders of the Arthropoda phylum, and Plantae, Protozoa, Bacteria, Virus and Fungi. The green number represents the top BLAST hits for species belonging to each of the clades. The red numbers represent the all the BLAST hits for that specific clade. The orders Mictacea Spelaeogriphacea and Thermosbaenacea were not accessed due to the lack of protein sequences within the database. The BLAST hits were performed with an e value of $1e^{-5}$ within the NCBI nr database.

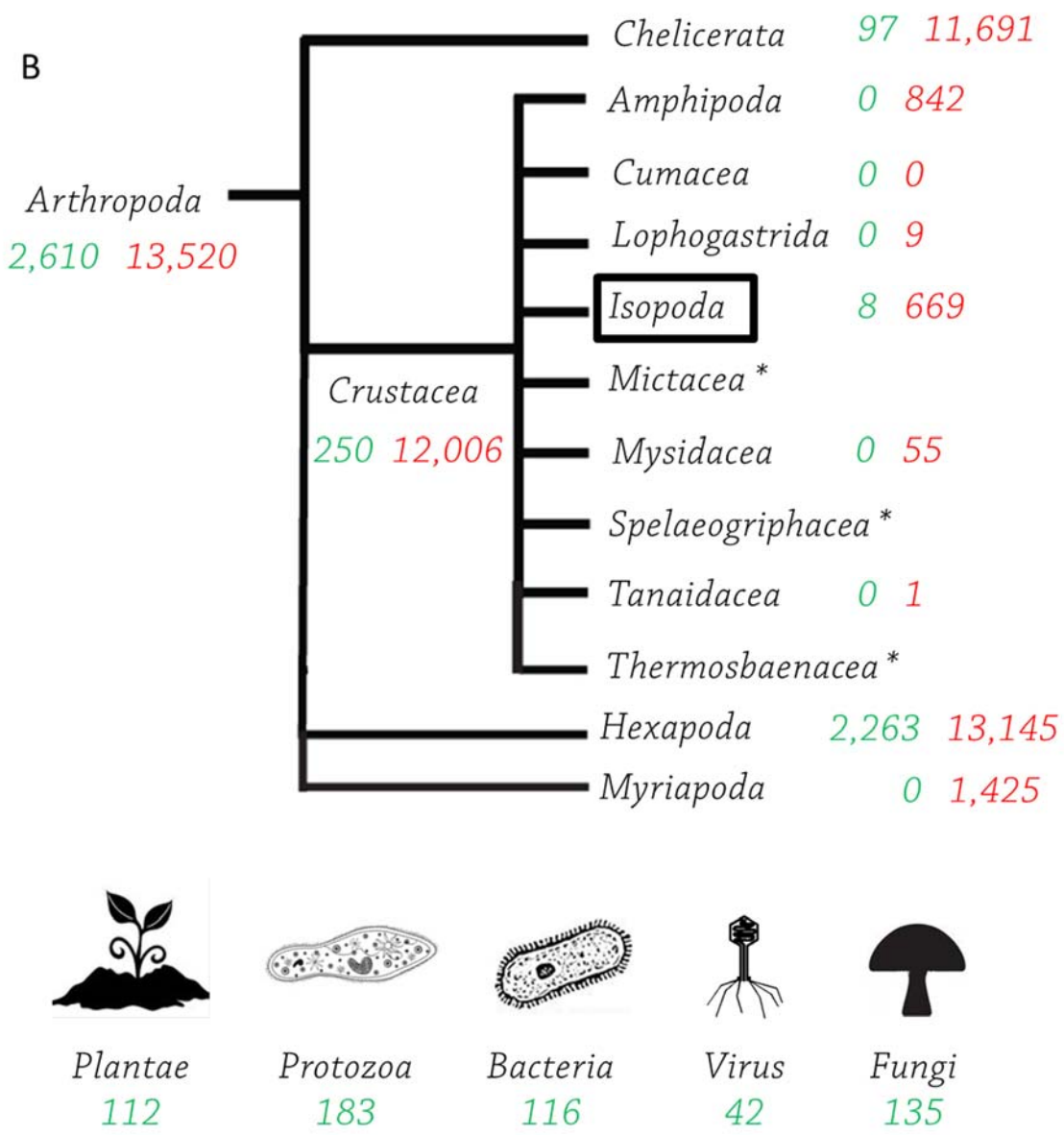

Fig 6.1 (cont.) Phylogenetic distribution of species of BLAST hits for the *Porcellionides pruinosus* transcriptome transcripts. A – BLAST hits for Metazoa phyla. B – Total number of BLAST hits for some subphyla and orders of the Arthropoda phylum, and Plantae, Protozoa, Bacteria, Virus and Fungi. The green number represents the top BLAST hits for species belonging to each of the clades. The red numbers represent the all the BLAST hits for that specific clade. The orders Mictacea Spelaeogriphacea and Thermosbaenacea were not accessed due to the lack of protein sequences within the database. The BLAST hits were performed with an e value of $1e^{-5}$ within the NCBI nr database.

The 10 Top-Hit species distributions is presented in Fig. 6.2, where it was depicted the species mainly belonging to the phylum Arthropoda, except for *Branchiostoma floridae* (Chordata), *Lottia gigantea* (Mollusca) and *Capitella teleta* (Annelida). The mean sequence distribution similarity was observed at 60%.

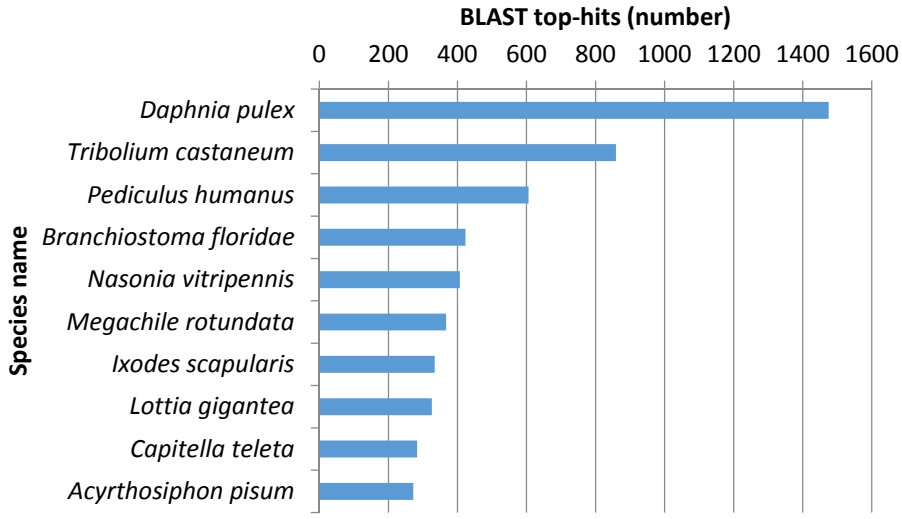

Fig. 6.2 The 10 Top-Hit species distribution.

For the GO analysis performed on the 13,438 transcripts, 11,280 transcripts (83.94%) could be assigned to a GO term (Fig. 6.3). A total of 45.16% of the transcripts were found to be involved in biological processes, including metabolic process (4,356 transcripts with percentages of 11.22%), cellular process (3,337; 8.59%), single-organism process (2,119; 5.46%), biological regulation (1,711; 4.41%), localization (1,371; 3.53%), response to stimulus (1,194; 3.07%), cellular component organization or biogenesis (829; 2.13%), developmental process (724; 1.86%), multicellular organismal process (647; 1.67%) and reproduction (204; 0.53%) as well as other activities (147; 0.38%). A total of 31.54% had potential molecular functions, including binding (6,290; 16.20%), catalytic activity (4,060; 10.46%), transporter activity (707; 1.82%), structural molecule activity (280; 0.72%), enzyme regulator activity (254; 0.65%); nucleic acid binding transcription factor activity (192; 0.49%), receptor activity (177; 0.46%), electron carrier activity (171; 0.44%) and other activities (115; 0.30%). GO analysis also showed that 23.30% were classified according to

a cellular component and could be divided into cell (4,288; 11.04%), organelle (2,267; 5.84%), macromolecular complex (1,309; 3.37%), membrane-enclosed lumen (522; 1.34%), membrane (328; 0.84%), extracellular region (305; 0.79%) and extracellular matrix (28; 0.07%).

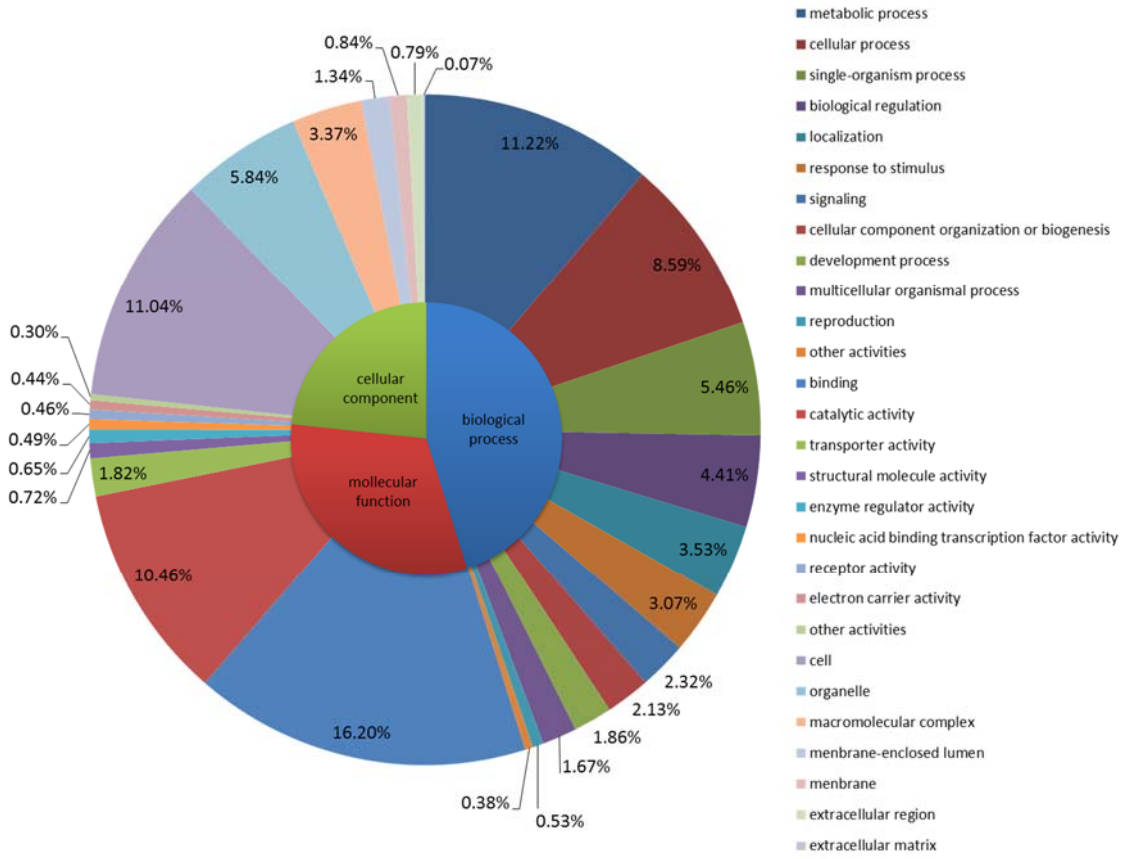

Fig. 6.3 Distribution of GO classifications of *Porcellionides pruinosus* into the three main categories: biological process, cellular component and molecular function and their subcategories.

The transcripts were associated with 119 predicted KEGG metabolic pathways (Fig. 6.4 and Fig. 6.5), and the number of transcripts in different pathways ranged from 1 to 203. The top 25 pathways with highest transcript numbers are shown on Table 6.3.

Table 6.3 Top 25 pathways with the highest transcript number.

No.	Pathway	Number of transcripts	Pathway ID
1	Purine metabolism	213	00230
2	Pyrimidine metabolism	88	00240
3	Aminoacyl-tRNA biosynthesis	49	00970
4	Phenylalanine metabolism	49	00360
5	Amino sugar and nucleotide sugar metabolism	47	00520
6	Oxidative phosphorylation	44	00190
7	Glutathione metabolism	42	00480
8	Lysine degradation	42	00310
9	Glycolysis / Gluconeogenesis	42	00010
10	Fatty acid degradation	40	00071
11	Thiamine metabolism	38	00730
12	Valine, leucine and isoleucine degradation	37	00280
13	Tryptophan metabolism	36	00380
14	Glycine, serine and threonine metabolism	36	00260
15	Carbon fixation pathways in prokaryontes	34	00720
16	Other glycan degradation	34	00511
17	Phenylpropanoid biosynthesis	34	00940
18	Arginine and proline metabolism	33	00330
19	Citrate cycle (TCA cycle)	33	00020
20	Phosphatidylinositol signalling system	33	04070
21	Butanoate metabolism	33	00650
22	Inositol phosphate metabolism	33	00562
23	Glycerolipid metabolism	31	00561
24	Methane metabolism	28	00680
25	Sphingolipid metabolism	28	00600

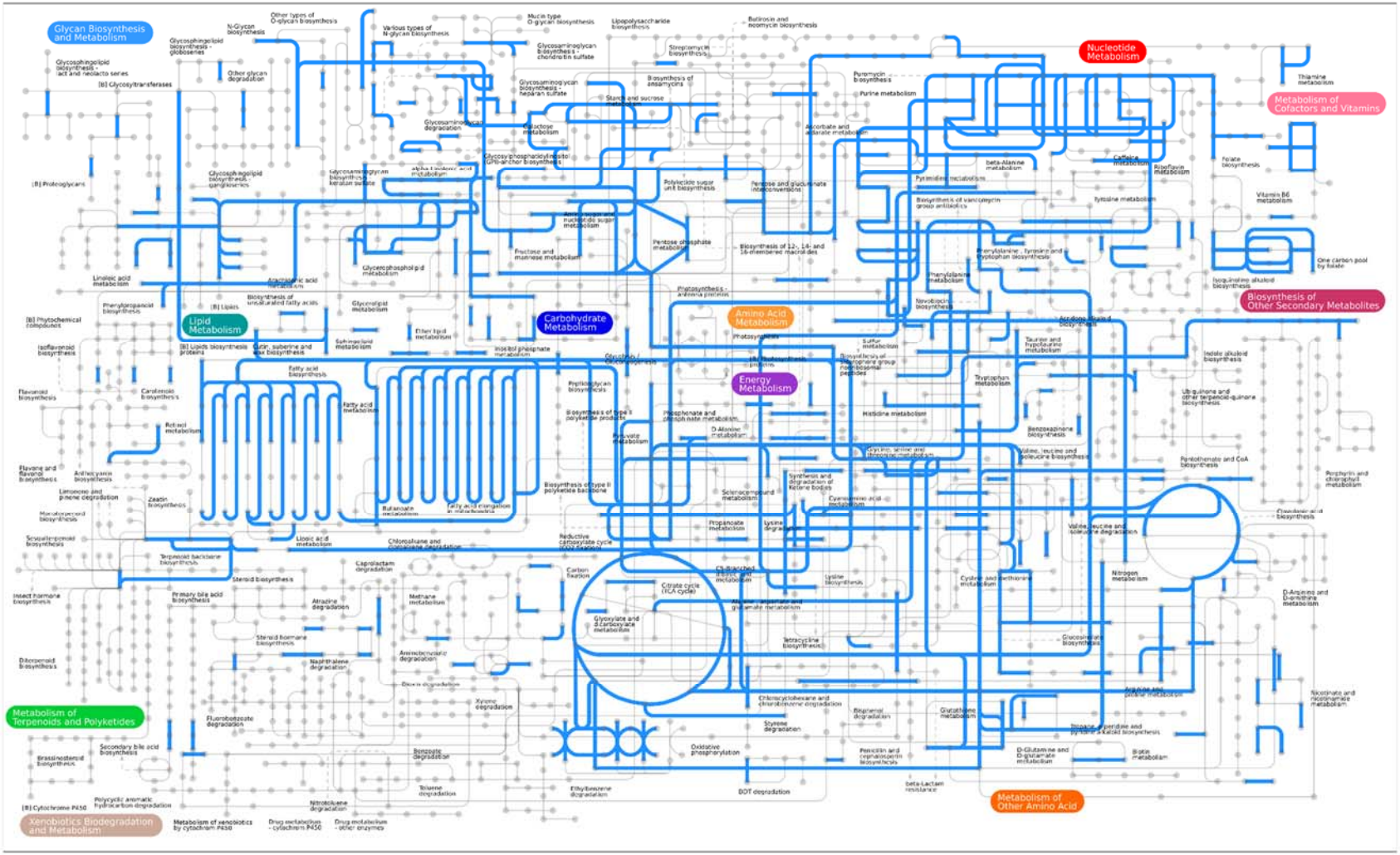

Fig. 6.4 General diagram of metabolic pathways identified in the *de novo* transcriptome of the terrestrial isopod *Porcellionides pruinosus*, extracted from the KEGG database (blue lines).

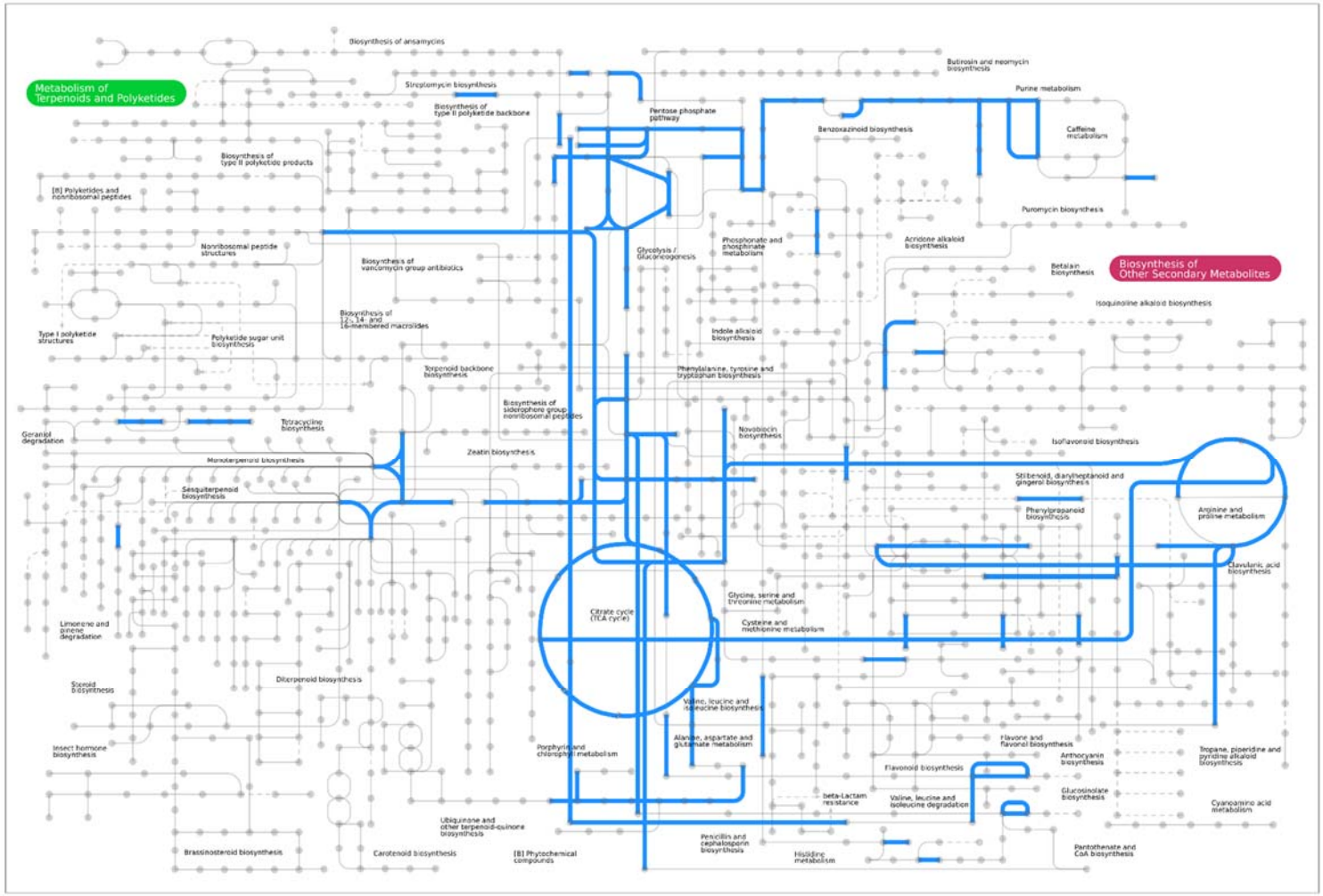

Fig. 6.5 General diagram of secondary metabolites biosynthesis pathways identified in the *de novo* transcriptome of the terrestrial isopod *Porcellionides pruinosus*, extracted from KEGG database (blue lines).

3.3. RNA-Seq analysis for the exposure to 50 mg Ni/kg soil

When isopods were exposed to 50 mg Ni/kg soil, the analysis derived from the KEGG pathway revealed that upregulated genes were related to a total of 46 pathways and downregulated genes were related to the impairment of a total of 42 pathways as shown in Fig. 6.6 and Fig. 6.7.

The top five annotated transcripts that suffer the most alterations for each concentration are depicted on Table 6.4.

Table 6.4 Top 5 downregulated and upregulated annotated transcripts for the exposure of *Porcellionides pruinosus* to 50mg Ni/kg soil.

Fold change	Uniprot ID	Gene homology
Downregulation		
-6.0667	P03646	Minor spike protein H
-6.0054	Q02357	Ankyrin-1
-5.8668	P69487	Scaffolding protein D
-5.7069	P07928	A' protein
-5.4525	P07932	Major spike protein G
Upregulation		
7.5577	O43451	Maltase-glucoamylase, intestinal
7.2203	Q9U572	Hemolymph cottable protein
6.6278	P91778	Alpha-amylase
6.5900	Q02157	Pancreatic triacylglycerol lipase
6.3992	O6P7A9	Lysosomal alpha-glucosidase

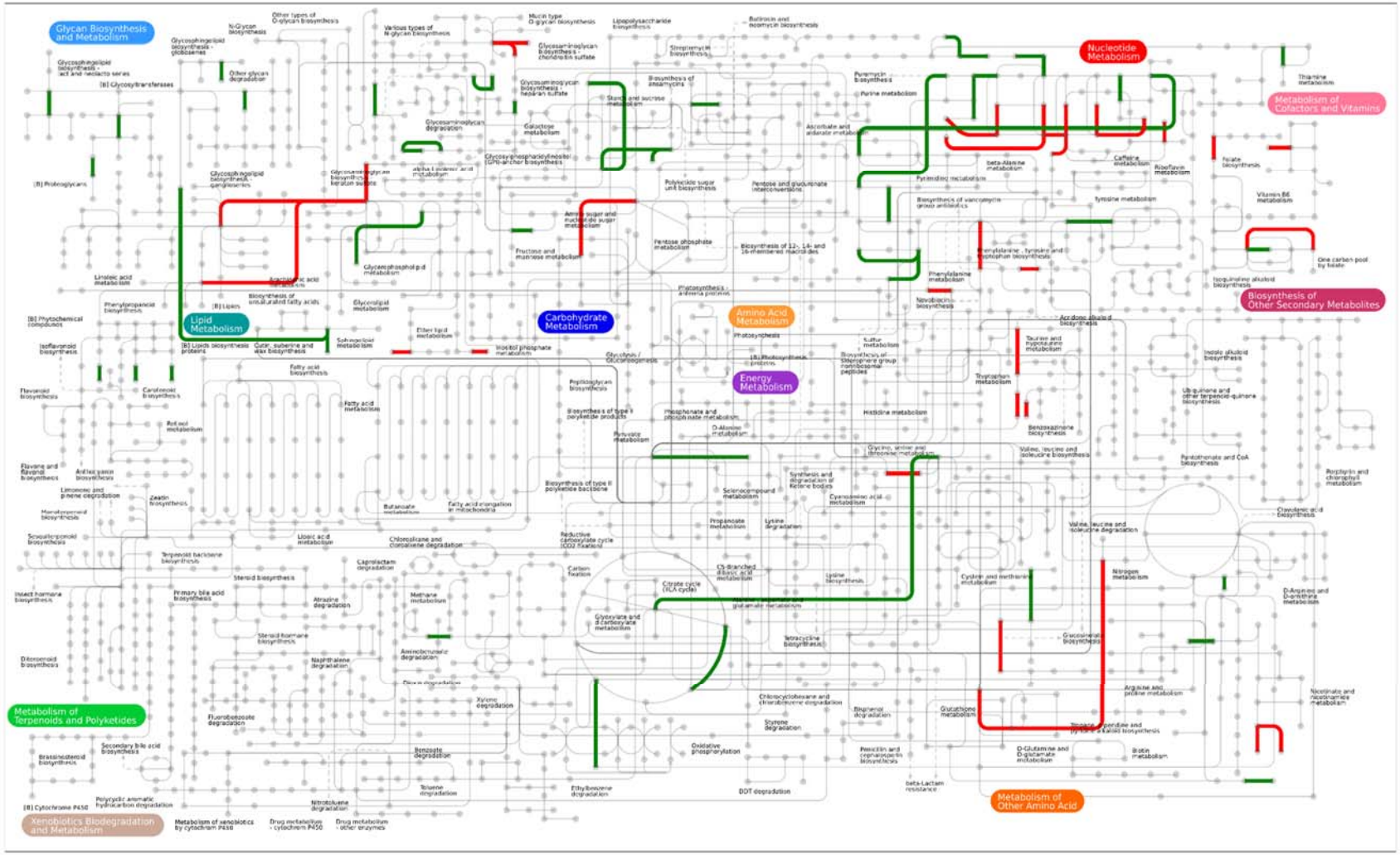

Fig. 6.6 General diagram of metabolic pathways of *Porcellionides pruinosus* being affected by the exposure to 50 mg Ni/kg soil extracted from the KEGG database. Green lines denote upregulated processes; Red lines denote downregulated processes.

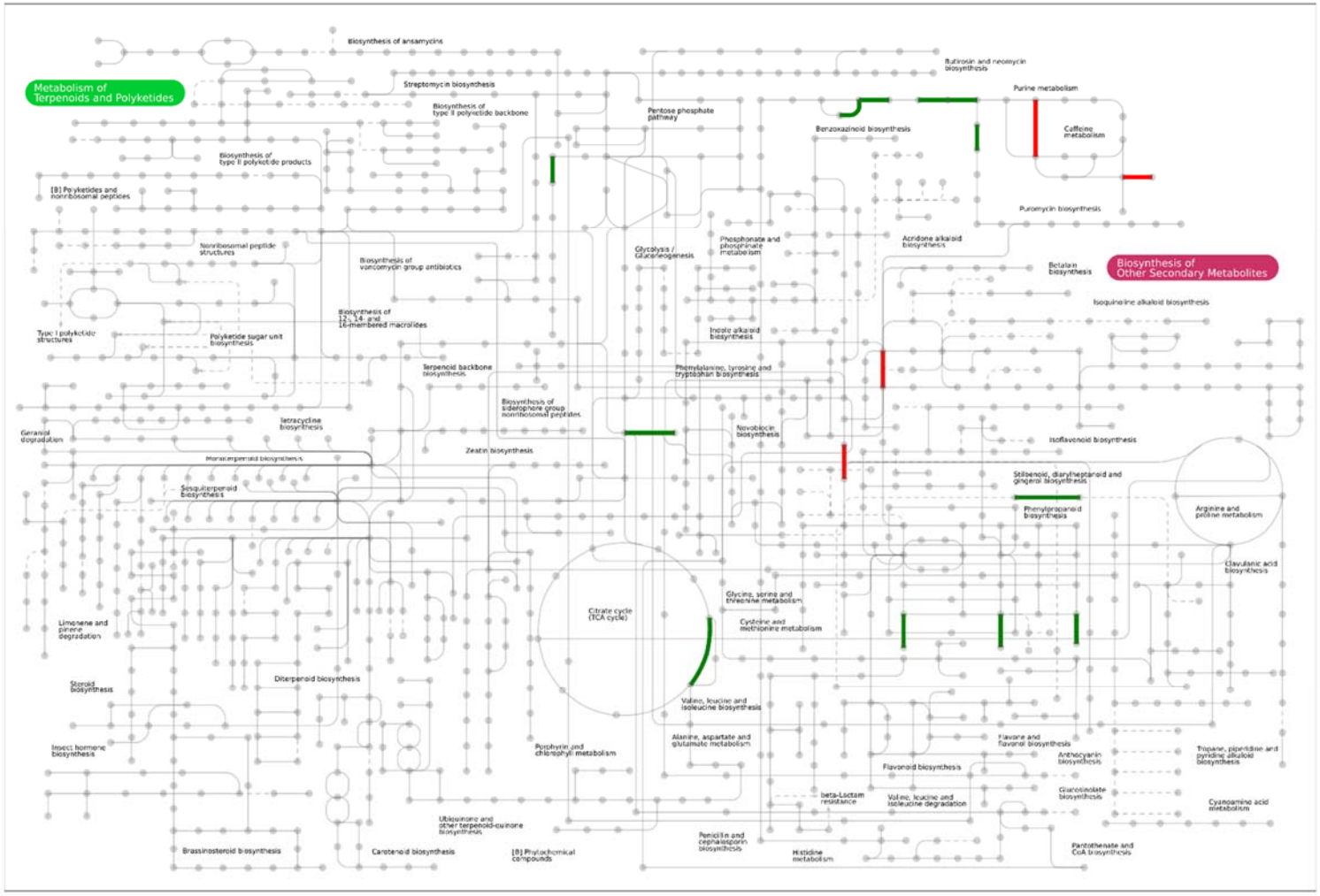

Fig. 6.7 General diagram of secondary metabolites biosynthesis pathways of *Porcellionides pruinosus* being affected by 50 mg Ni/kg soil extracted from the KEGG database. Green lines denote upregulated processes; Red lines denote downregulated processes.

The transcripts for the exposure to the lower concentration of nickel (50 mg/kg soil - Fig. 6.8) showed a similar distribution between up and downregulated processes. For biological process, the cellular component organization or the biogenesis and localization were the processes that showed more differences between up and down transcription. As for the molecular function, upregulated transcripts did not include receptor activity whereas downregulated transcripts did not include molecular transducer activity, protein tag or translation regulator activity. In the cellular components transcripts showed also very similar distributions.

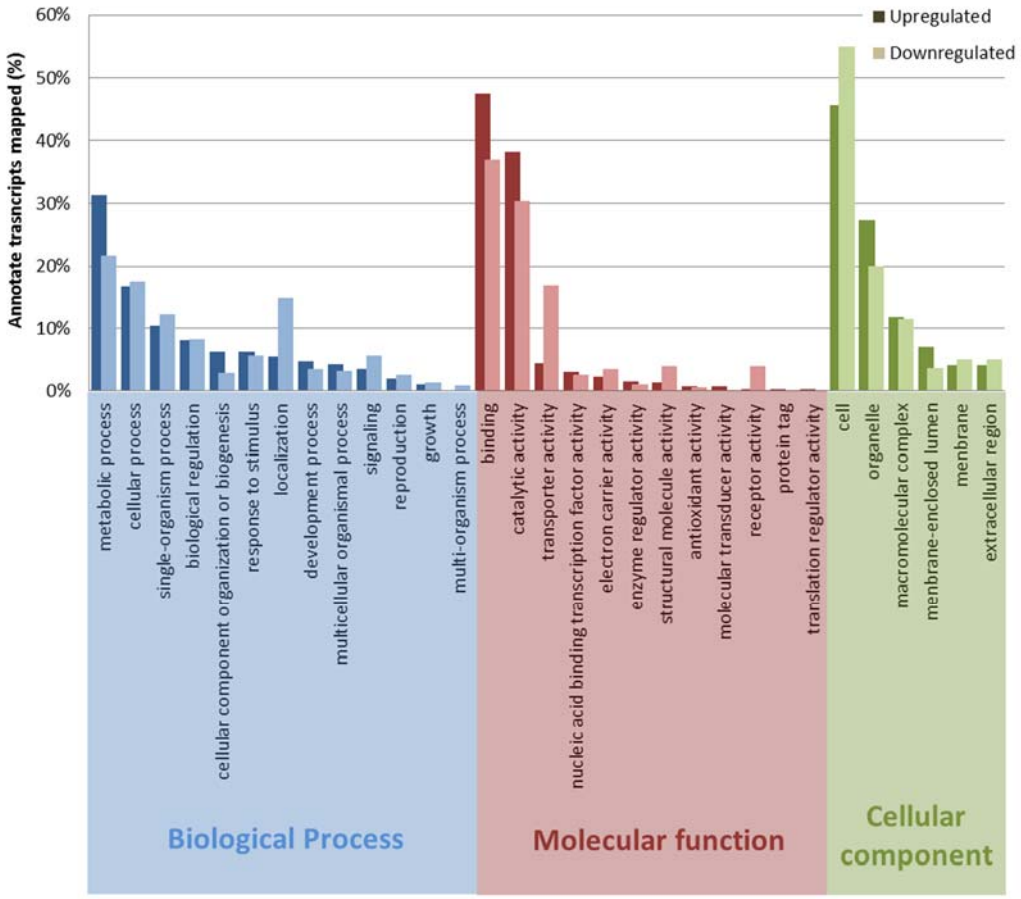

Fig. 6.8 Distribution of GO classifications of *Porcellionides pruinosus* regarding significant up and downregulated transcripts into three main categories: biological process, cellular component and molecular function and their subcategories. Column heights are the percentage of annotated transcripts that mapped to each correspondent GO term. Darker bars represent upregulated transcripts and lighter bars represent downregulated transcripts.

The GO enrichment analysis network is presented in Fig. 6.9 to Fig. 6.11. In a brief description, the upregulated biological processes for the exposure to 50 mg Ni/kg soil was 64% related to nucleosome assembly, 20% to sexual reproduction, 10% to membrane lipid metabolism and 6% to regulation of mRNA stability and others. As for the upregulated cellular components, 43% was related to lysosomes, 15 % to external encapsulating structure and 42% to other cellular components. Finally, the upregulated molecular functions are mainly distributed between carboxylpeptidase activity (30%), zinc ion binding (19%) and the incorporation/reduction of oxygen (16%). There were also 7% related to heme binding, 6% to single-stranded RNA binding, 6% to cyclin binding and 16% to other molecular functions. Regarding the downregulated biological processes for the exposure to 50 mg Ni/kg soil the majority was related to ion transport (79%), followed by positive regulation of protein kinase activity (17%) and other biological processes (4%). For the cellular component analysis the majority were related to a diverse number of cellular components (62%), but 17% were related to integral to plasma membrane, 16% to axoneme and 5% to the extracellular region part.

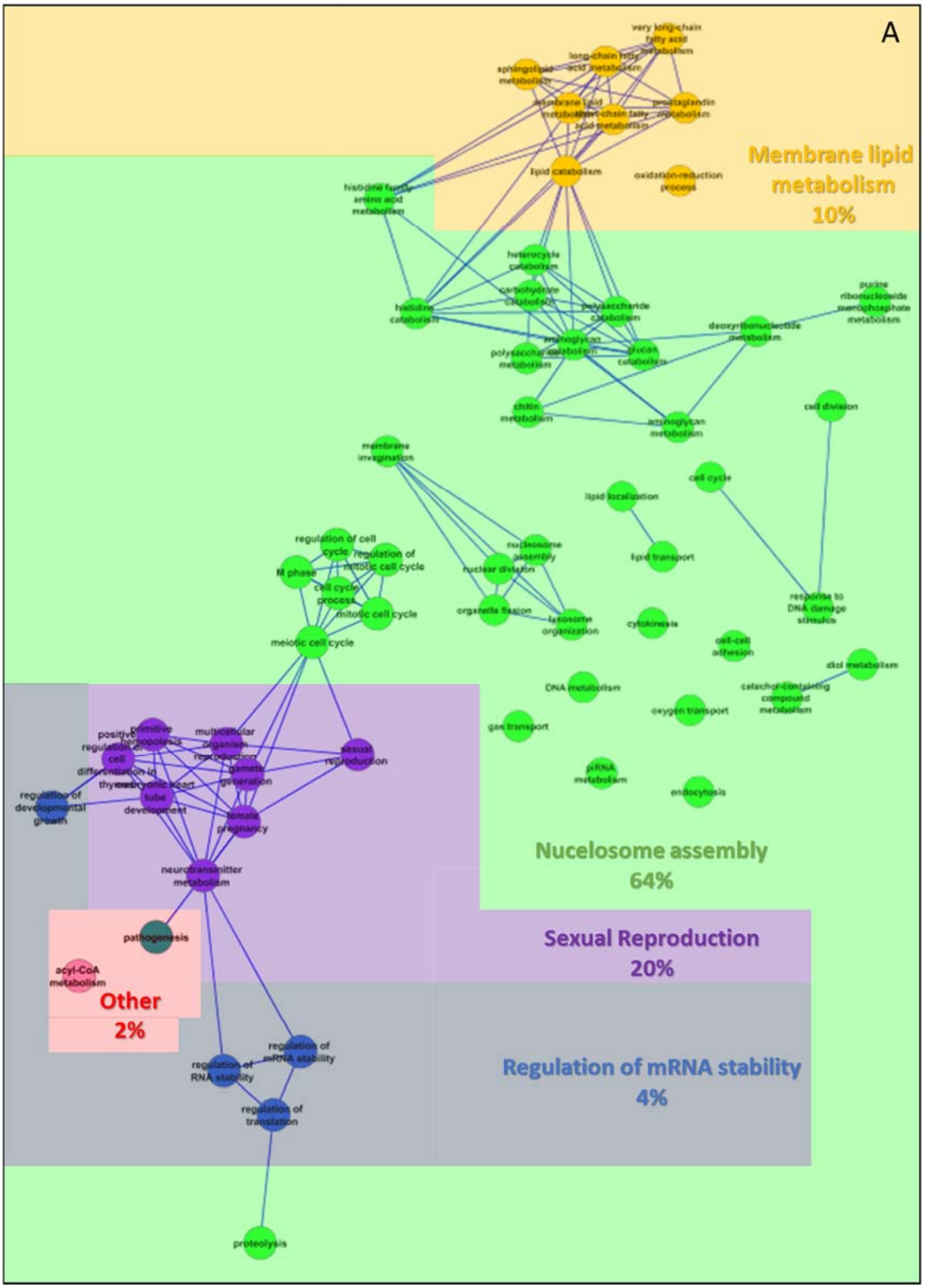

Fig. 6.9 GO enrichment analysis network of upregulated transcripts for *Porcellionides pruinosus* exposed to 50 mg Ni/kg soil. A- Biological Process, B- Cellular Component; C- Molecular Function

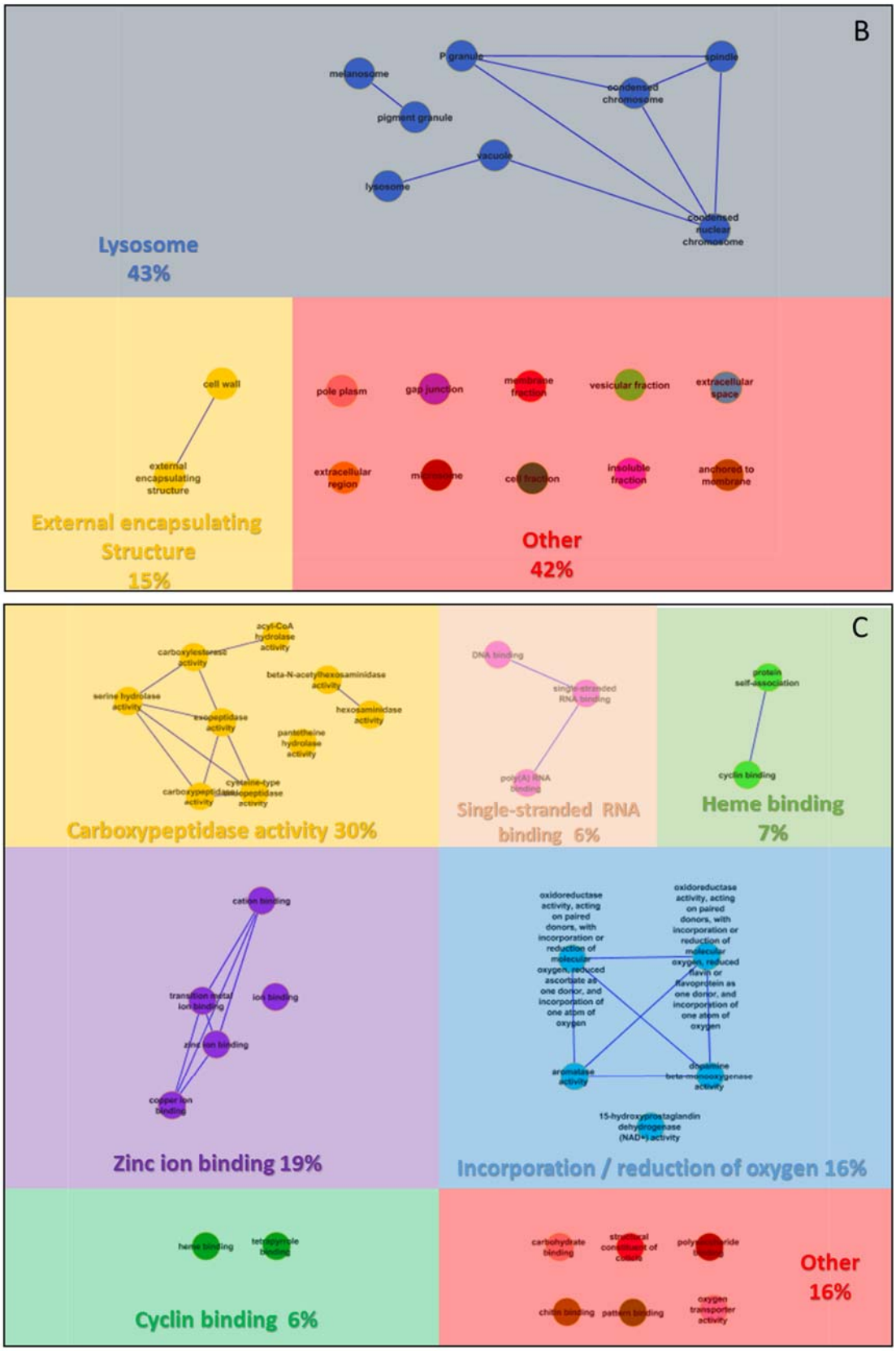

Fig. 6.10 GO enrichment analysis network of upregulated transcripts for *Porcellionides pruinosus* exposed to 50 mg Ni/kg soil. A- Biological Process, B- Cellular Component; C- Molecular Function.

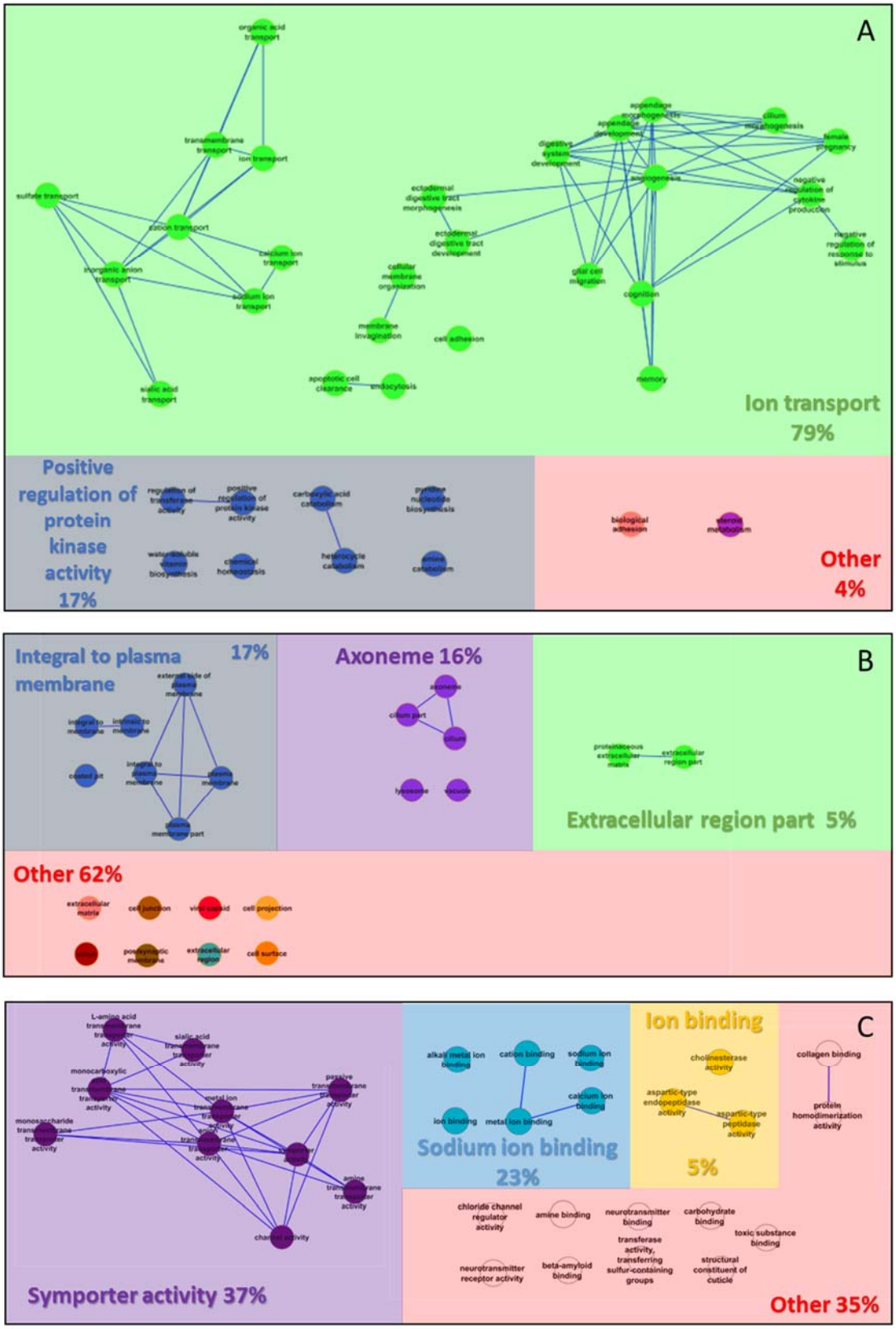

Fig. 6.11 GO enrichment analysis network of downregulated transcripts for *Porcellionides pruinosus* exposed to 50 mg Ni/kg soil. A- Biological Process, B- Cellular Component; C- Molecular Function.

3.4. RNA-Seq analysis for the exposure to 250 mg Ni/kg soil

When isopods were exposed to 50 mg Ni/kg soil, the analysis derived from the KEGG pathway revealed that upregulated genes were related to a total of 41 pathways and downregulated genes were related to the impairment of a total of 28 pathways as shown in Fig. 6.12 and Fig. 6.13.

The top five annotated transcripts that suffer the most alterations for each concentration are depicted on Table 6.5.

Table 6.5 Top 5 downregulated and upregulated annotated transcripts for the exposure of *Porcellionides pruinosus* to 250mg Ni/kg soil.

Fold change	Uniprot ID	Gene homology
Upregulation		
-6.4815	P07932	Major spike protein G
-6.0973	P03641	Capsid protein F
-5.8099	P07928	A' protein
-5.6372	Q6NUT3	Major facilitator superfamily domain containing protein 12
-5.6175	P03646	Minor spike protein H
Downregulation		
8.7822	Q86T26	Transmembrane protease serine 11B
8.1253	P20214	Neuroglian
6.6222	Q0IIH7	Suppressor tumorigenicity 14 protein homolog
6.5738	Q5RAG8	Prolyl 4-hydroxylase subunit alpha-1
6.3503	Q9W5U2	Chitinase 3

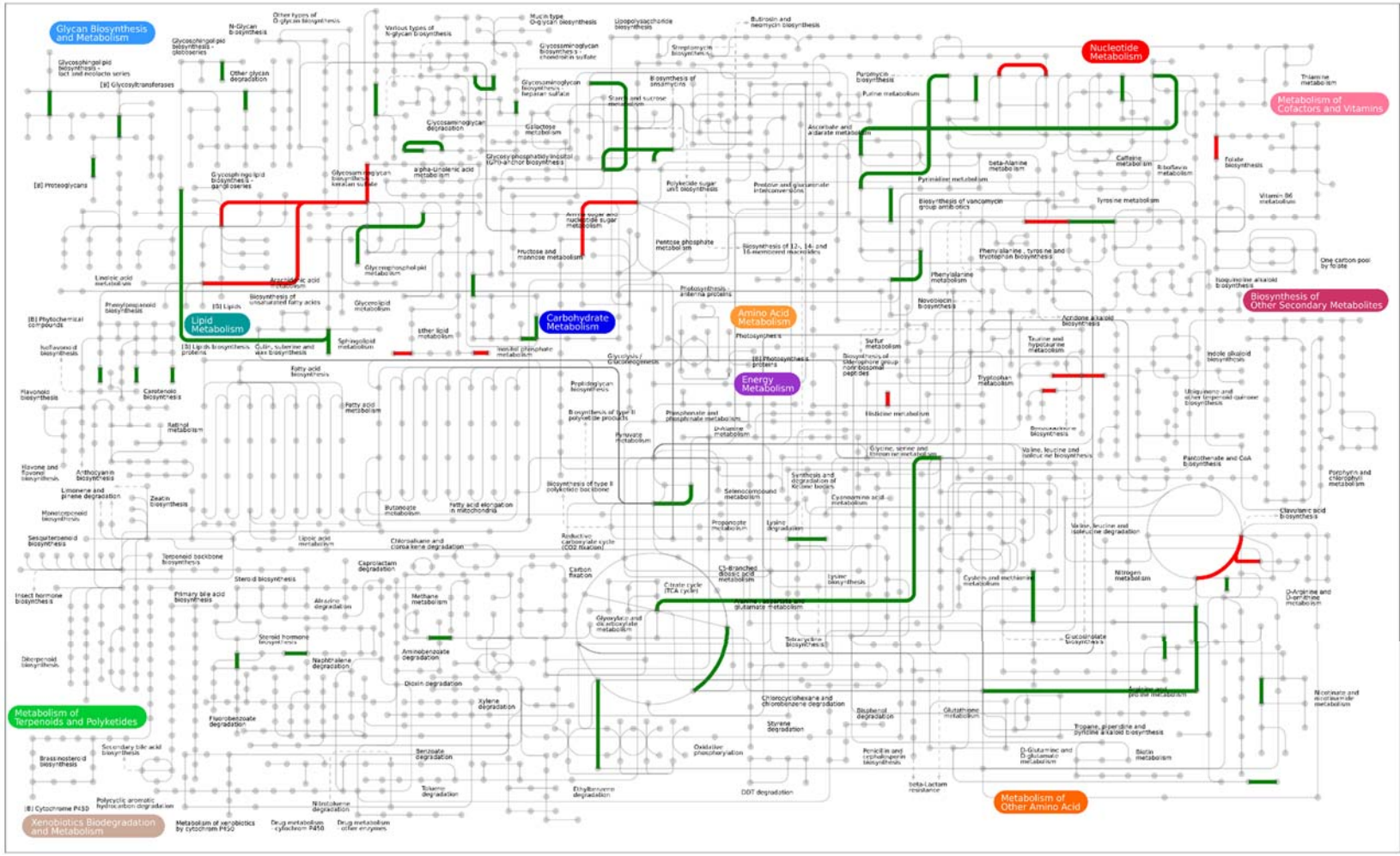

Fig. 6.12 General diagram of metabolic pathways of *Porcellionides pruinosus* being affected by the exposure to 50 mg Ni/kg soil extracted from the KEGG database. Green lines denote upregulated processes; Red lines denote downregulated processes.

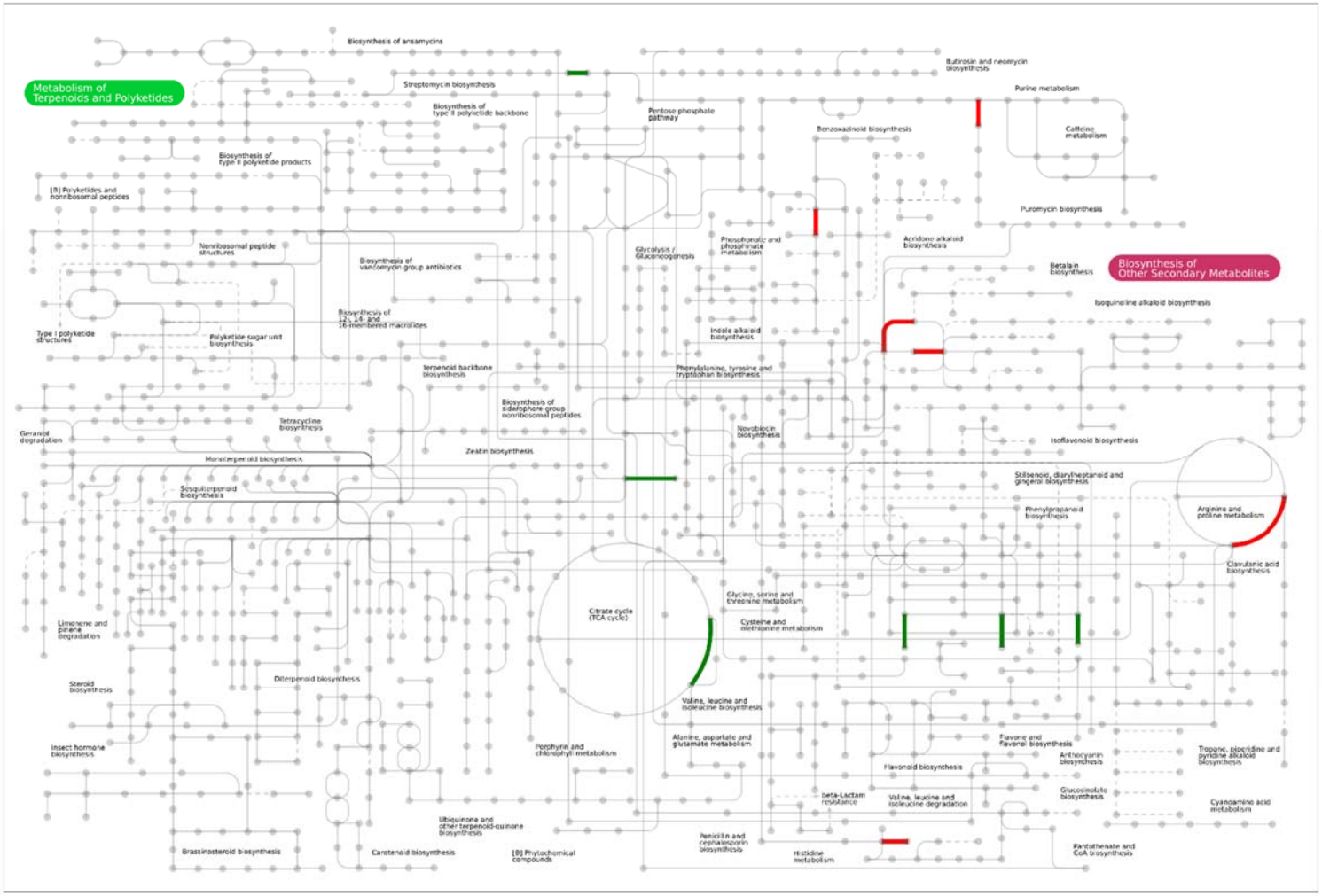

Fig. 6.13 General diagram of secondary metabolites biosynthesis pathways of *Porcellionides pruinosus* being affected by the exposure to 50 mg Ni/kg soil extracted from the KEGG database. Green lines denote upregulated processes; Red lines denote downregulated processes.

The transcripts for the exposure to the higher concentration of nickel (250 mg/kg soil - Fig. 6.14 show a similar distribution between up and downregulated processes. For biological process, the biological regulation and localization were the processes that showed more differences between up/down transcription. As for the molecular function, downregulated transcripts do not include molecular transducer activity or protein tag, also binding, catalytic activity, transporter activity and, structural molecule activity, and electron carrier activity are the most different. The cellular components show also very similar distributions, except for the extracellular matrix that does not appear in the downregulated transcripts.

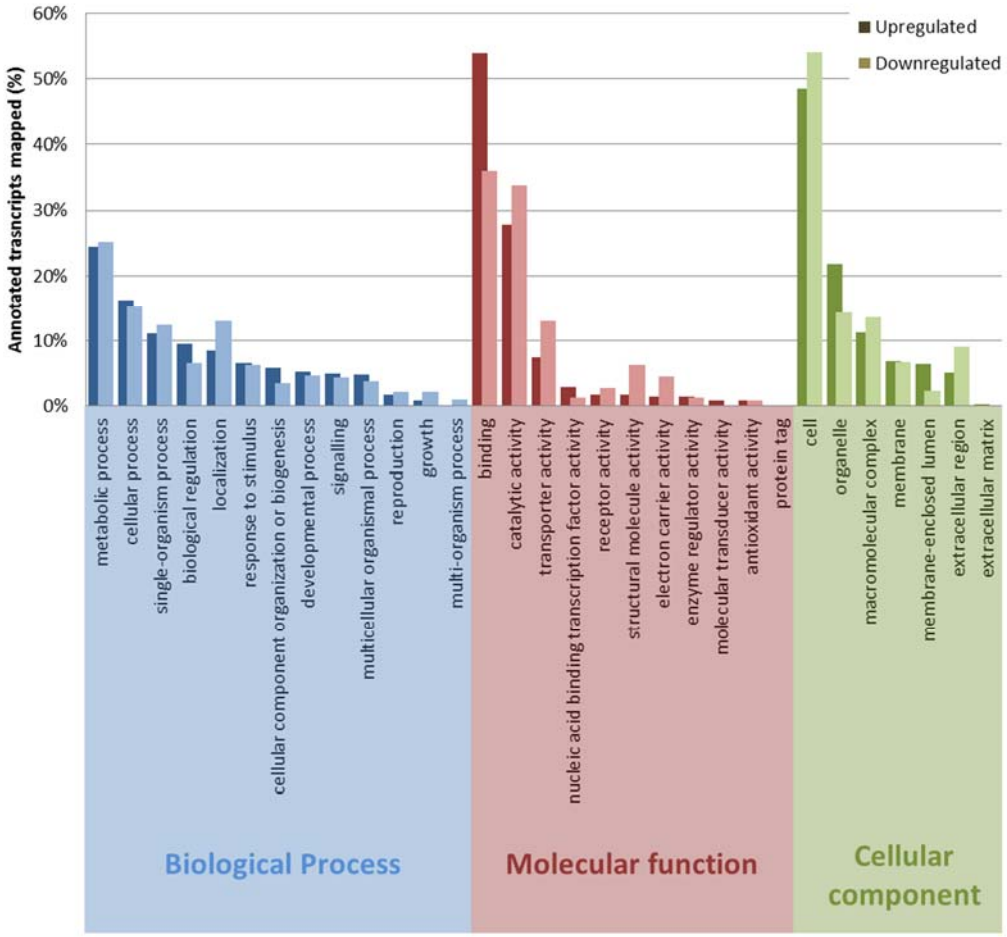

Fig. 6.14 Distribution of GO classifications of *Porcellionides pruinosus* regarding significant up and downregulated transcripts for organisms exposed to 250 mg/kg soil into three main categories: biological process, cellular component and molecular function and their subcategories. Column heights are the percentage of annotated transcripts that mapped to each correspondent GO term. Darker bars represent upregulated transcripts and lighter bars represent downregulated transcripts.

The GO enrichment analysis network is presented in Fig. 6.15 and Fig. 6.16. The upregulated biological processes for the exposure to 250 mg Ni/kg soil were 45% related to sexual reproduction, 37% to nucleosome assembly, 10% to histidine family metabolism and 8% to regulation of mRNA stability and others. For the upregulated cellular components, 24% were related to external encapsulating structure, 29% anchored to membrane, 17% to phosphate granule and 30% to other cellular components. Finally, the upregulated molecular functions are mainly distributed between zinc ion binding (31%), serine hydrolase activity (21%), symporter activity (10%) and 38% to other molecular functions. Regarding the downregulated biological processes for the exposure to 250 mg Ni/kg soil the majority was related to ion transport (73%), followed by plasma membrane part (27%) and other biological processes (21%). For the cellular component analysis the majority were related to other molecular functions (43%), followed by symporter activity (32%) and serine type peptidase activity (25%).

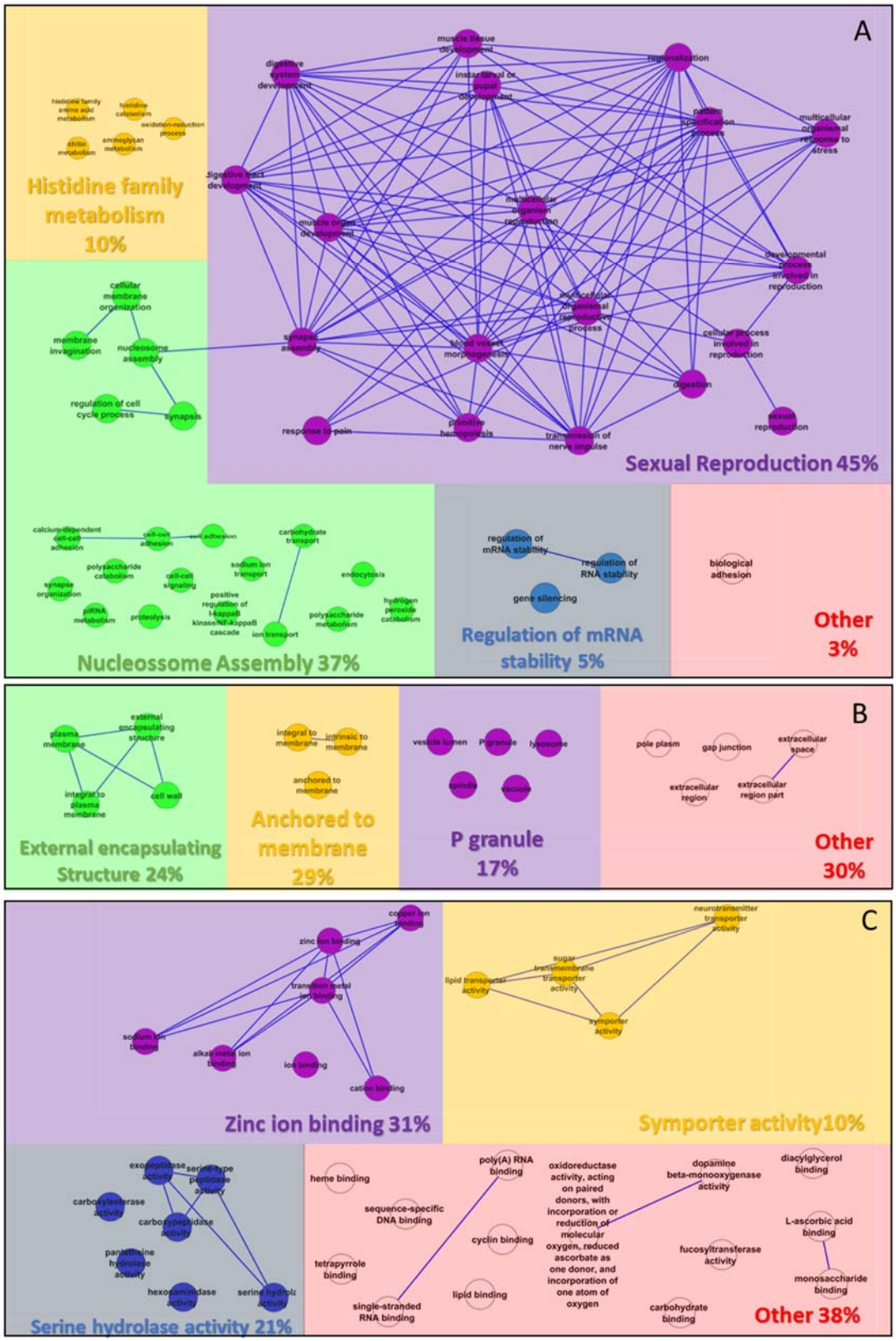

Fig. 6.15 Go enrichment analysis network of upregulated transcripts for organisms belonging to the species *Porcellionides pruinosus* exposed to 250 mg Ni/kg soil. A- Biological Process, B- Cellular Component; C- Molecular Function.

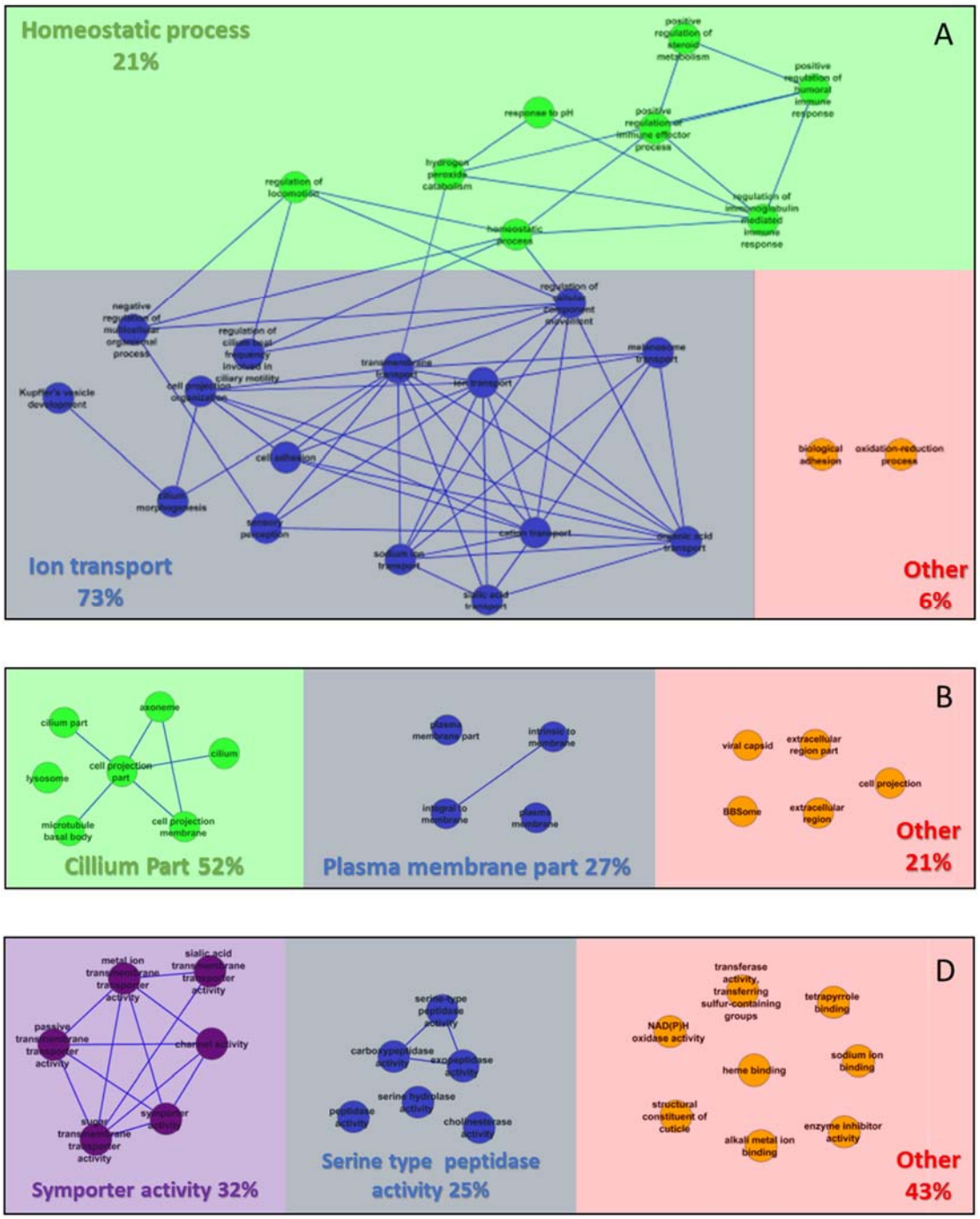

Fig. 6.16 Go enrichment analysis network of downregulated transcripts for organisms belonging to the species *Porcellionides pruinosus* exposed to 250 mg Ni/kg soil. A- Biological Process, B- Cellular Component; C- Molecular Function.

3.5.RNA-Seq analysis: nickel treatment exposures comparison

In Fig. 6.17 the RNA-Seq analysis for the exposures to nickel is presented, and the up/downregulated annotated transcripts unique and common to both concentrations (50 mg and 250 mg/kg soil) and respective GO classifications. The distribution between the up and downregulated transcriptions showed a big differentiation between biological process, and molecular function as upregulated transcripts are more distributed into the second category and the downregulated transcripts into the first category. A closer look shows that cellular process, cellular component organization or biogenesis, localization (biological process), binding, structural molecular activity, receptor activity, molecular transducer activity, antioxidant activity, protein tag (molecular function), membrane enclosed lumen and extracellular region (cellular component) are the main differences. In the specific case of molecular transducer activity, antioxidant activity and protein tag are not represented in the downregulated transcripts.

The KEGG pathway analysis revealed that upregulated genes impacted a total of 29 pathways and downregulated genes impacted a total of 13 pathways as shown in Fig. 6.18 and Fig. 6.19.

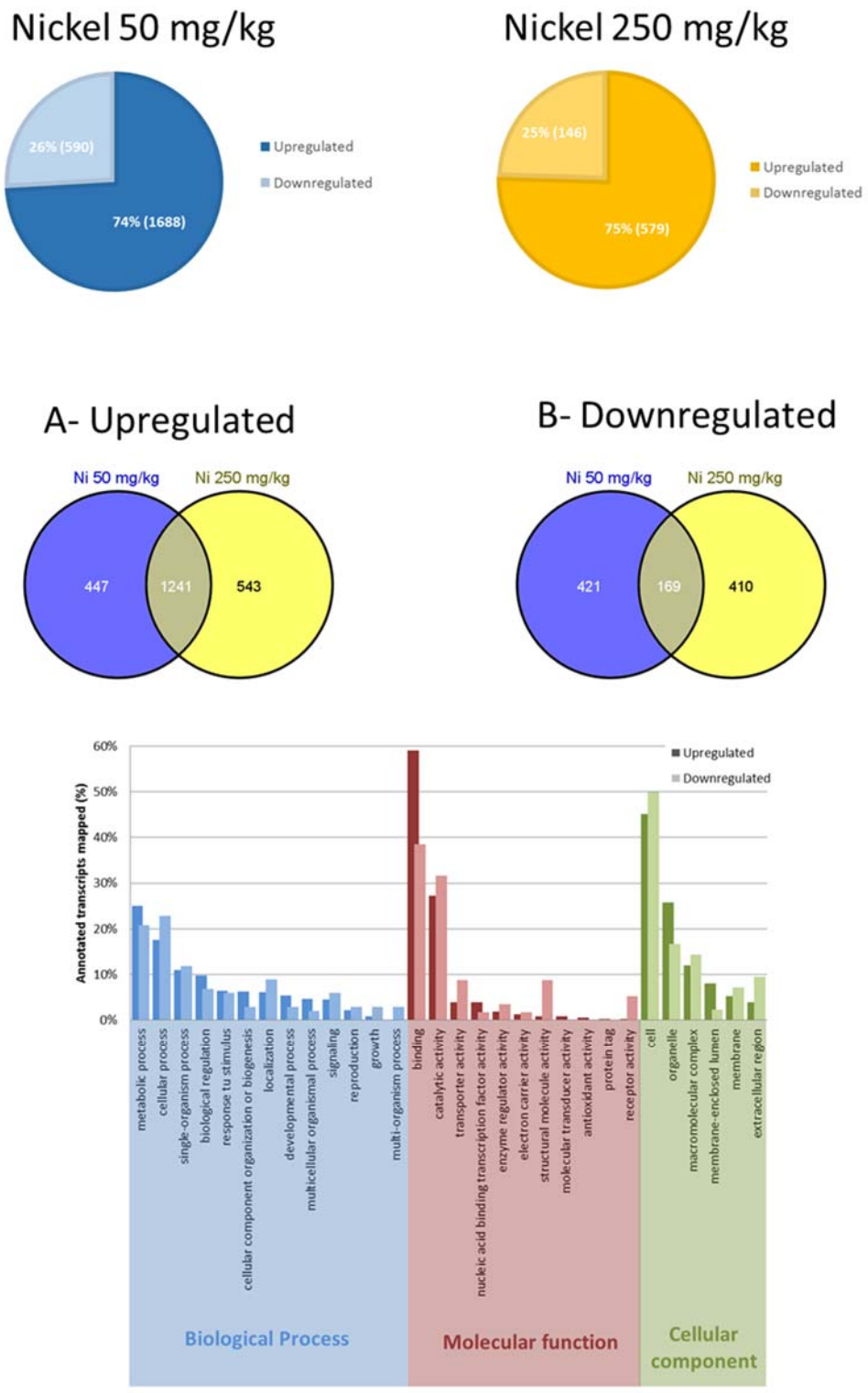

Fig. 6.17 Nickel exposures to 50 and 250 mg/kg soil and respective up and downregulated transcripts along with Venn diagrams' showing the differentially expressed transcripts that are unique and common between treatments (A- upregulated transcripts; B- downregulated transcripts). Diagram with the distribution of GO classifications of significant up and downregulated transcripts into three main categories: biological process, cellular component and molecular function and their subcategories. Upregulated transcripts are always assigned into dark colors and downregulated transcripts are always assigned into lighter colors. Transcript numbers always represent annotated transcripts.

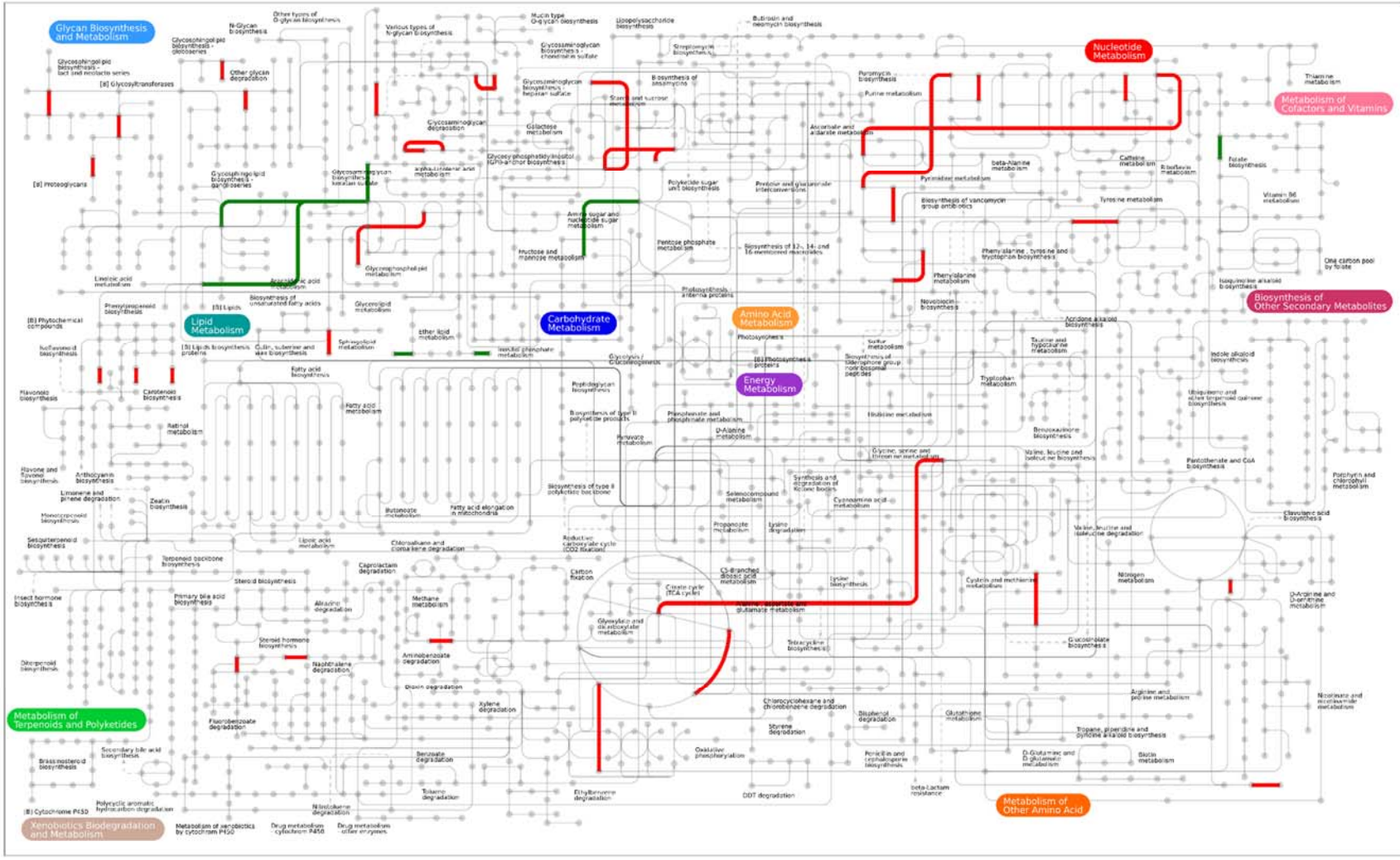

Fig. 6.18 General diagram of common metabolic pathways of *Porcellionides pruinosus* being affected by Ni extracted from the KEGG database. Green lines denote upregulated processes; Red lines denote downregulated processes.

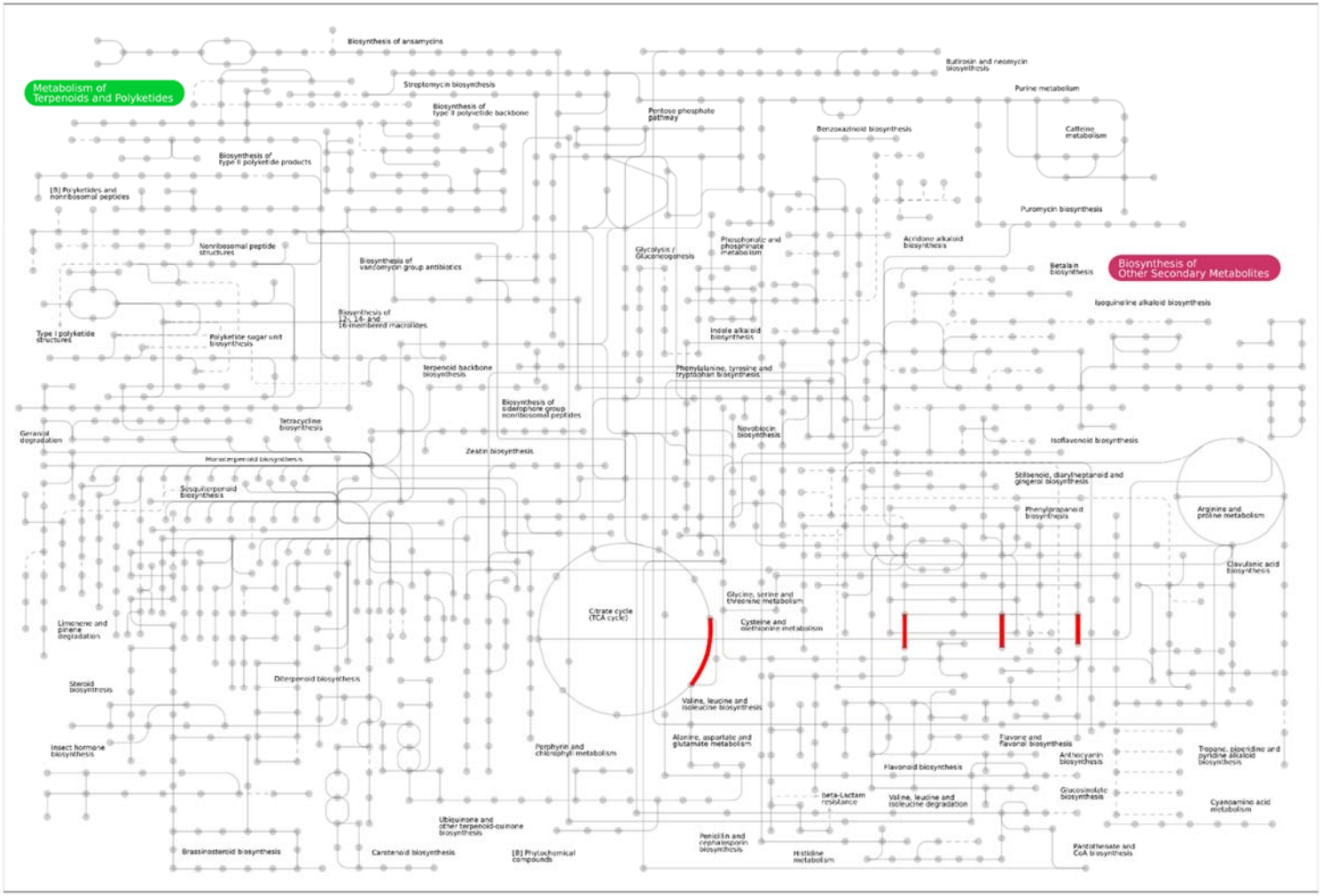

Fig. 6.19 General diagram of secondary metabolites biosynthesis pathways of *Porcellionides pruinosus* being affected by the exposure to 50 mg Ni/kg soil extracted from the KEGG database. Green lines denote upregulated processes; Red lines denote downregulated processes.

4. Discussion

This work presents the first full transcriptome for a terrestrial isopod, belonging to the species *P. pruinosus*. Until the present, the order Isopoda was one of the most uncharacterized orders regarding genomic data, and just by performing a quick search thought the NCBI database approx. a total of 4,000 proteins were shown, almost all belong to mitochondrial genes, mainly cytochrome oxidase I (COI) used for barcoding. Here we present a high number of genes belonging to a diverse number of pathways as can be observed from the overall KEGG's pathway maps, and GO classification figures. The GO analysis of this new transcriptome shows approx. 80% similarity to Arthropoda and approx. 20% to other phyla, which sustains not only a robust assembly, but also a higher similarity to other species of the same phylum, proving also to be an important tool for further studies such as phylogenetic similarities. Although some caution should be taken when observing the top-hit species similarity distribution to our transcriptome (since species with more sequences in the database will have more probability to appear as first hit) the high number of hits to the crustacean *Daphnia pul*ex (an aquatic and wide used species for aquatic ecotoxicology, along with the species *Daphnia magna*) makes future studies based in comparisons between these two ecological relevant and different species perfect for the development and better understanding of toxicological pathways.

The KEGG's pathways that can be analysed using this new transcriptome represent all the major pathways of an organism (Fig. 6.4). The exception are the pathways related to the "metabolism of terpenoids and polyketides", mainly observed in plants (e.g. Bohlmann et al. 1998) although also reported for several insects like termites (e.g. Moore, 1968), and related to "xenobiotic degradation and metabolism", pathways that should not be affect by the exposure to metals and thus making sense the low coverage of genes that are included in these pathways. Another important feature to pinpoint is the even distribution within the three main GO classifications (biological processes, cellular component and molecular function) once again highlighting the importance of this transcriptome and the high information provided, allowing its use in further studies.

The second part of our study focuses on the toxicological effects of the micro-essential metal nickel and the regulation of several important pathways that are being impacted. As expected

the majority of the analysed transcripts showed a dose-dependency among Ni concentrations and the transcription observed. Although Ni is a recognized carcinogenic metal, its molecular mechanisms and physiological alterations are still poorly known. The present transcriptome showed several distinctive toxicological impacts that ranged from genetic and epigenetic changes, ion trafficking and storage and even neural and physiological impairment as described below. A special note should also be included regarding the activation of hypoxia signalling mediated by the hypoxia inducible factor 1 (HIF) that *per se* interacts with the von Hippel Lindau tumour suppressor protein (VHL). Although a vast number of studies reported nickel as a metal producing very specific patterns of gene expression that are similar to hypoxia responses (Maxwell and Salnikow, 2004; Salnikow et al., 2000; Salnikow et al., 2003), our results showed almost no transcription alteration on those genes suggesting that at least in terrestrial isopods this response is not present or that the exposure concentrations were not high enough to activate this pathways as also suggested in Chapter IV.

4.1. Genetic and epigenetic impact

DNA damage, cell cycle impairment and death were processes highlighted as induced by Ni exposure and have already been reported previously (e.g. Ahamed et al., 2011; Hartwig et al., 2002; Kasprzak et al., 2003; Shiao et al., 1998). The main mechanism underlying these impairments are related to DNA repair inhibition and/or pro-apoptosis systems as DNA polymerase malfunction causes base mis-incorporation into the new synthesized oligonucleotides (Sirover and Loeb, 1976). Identical processes were identified within our study, the DNA repair and recombination proteins RAD54 and DNA double-strand break repair RAD50 appear 2 - 4 fold upregulated for both exposure concentrations. The E3 ubiquitin protein ligase RAD18 required for post-replication repair of UV-damaged DNA appears more than 2 fold upregulated, in fact its upregulation underlies the impact of reactive oxygen species (ROS) as a similar toxicological mechanism involved in Ni and UV radiation toxicity described below. Along with the previous types of proteins, the mismatch repair endonuclease PMS2 also appears 2.09 and 0.74 upregulated respectively for 50 mg and 250 mg Ni/kg soil exposures. PMS2 not only acts within DNA repair but is also implicated in

DNA damage signalling, a process which induces cell cycle arrest and can lead to apoptosis in case of major DNA damages. This which can explain a lower upregulation for organisms exposed to the higher concentration of Ni. In fact an inhibition of apoptosis and impairment in cell cycle was observed, thus showing that Ni not only alters and mismatches DNA, but also prevents important "checkpoint mechanisms" like the replication of these abnormal cells. The apoptosis inhibitor IAP, responsible for the inhibition of cell apoptosis and also involved in copper homeostasis (please see below ion trafficking and storage), appears 2.5 - 3.5 fold upregulated in both exposure concentrations. G2 mitotic-specific cyclin-A and cyclin-B types along with mitotic spindle assembly checkpoint MAD2A and the mitotic checkpoint serine/threonine protein kinase BUB1, responsible for diverse functions during cell division are also impacted appearing 2 - 3 fold upregulated. Regarding this, the BUB1 has also been related with several forms of cancer: gastric cancer (Grabsch et al., 2003), breast cancer (Myrie et al., 2000), lung cancer (Haruki et al., 2001) and thyroid cancer (Ouyang et al., 2002), showing again the carcinogenic effect of Ni.

The results obtained also imply epigenetic changes. The changes observed are closely connected with oxidative stress induced by metals and it can be considered a unifying process across different metal exposures explained below (Valko et al., 2005). Regarding specifically Ni it has been proposed that it triggers *de novo* DNA methylation, induces hypermethylation, enhances chromatin condensation and may even replace magnesium in DNA interactions (Baccarelli and Bollati, 2009; Lee et al., 1995; Salnikow and Zhitkovich, 2007). Here upregulation in genes encoding histones was observed ranging from 2 - 3.5 fold and mainly in histone H1 and H2 (A/B) but also the histone lysine *N*-methyltransferase SETD7 used as a specific tag for epigenetic transcriptional activation. Another important upregulated transcript is the chromatin assembly factor 1, subunit B (CHAF1B) which required for the assembly of histone octamers onto newly-replicated DNA, but also plays a role in mediate chromatin assembly and in DNA replication and DNA repair already described above. Finally, DNA (cytosine-5-)-methyltransferase 1 (DNMT1) or methylated-DNA-protein-cysteine methyltransferase transcripts also appeared 2 - 3 fold upregulation indicating the hypermethylation already observed in other studies (e.g. Hermann et al., 2004; Sutherland et al., 2001).

4.2. Ion trafficking and storage

As stated previously terrestrial isopods are organisms that can assimilate high amounts of metals from the environment (Donker et al., 1990; Drobne, 1997; Hopkin, 1990) by their specific compartmentalisation of metals into specialized '*B*' and '*S*' cells of the hepatopancreas (Hopkin and Martin, 1982). But although these cells will be the final destiny for nickel storage and/exclusion in terrestrial isopods, specific proteins responsible for uptaking, trafficking and distribution and/or nickel storage are not known for animals. Nickel is described has a metal that will use pre-existent (and specific) proteins used by other transition metals, as for example zinc transporters, for its own transport (Sterling et al., 2007). Within our study we found two separate but well identified patterns that can be related to Ni trafficking, storage and/or excretion and that fits within the proposed 'Ahearn Model' described by Ahearn (2010). In brief the trafficking of Zn into lysosomes is performed by a pH gradient (low pH inside, high pH outside) that will involve membrane-bound, ATP-dependent H^+ or Zn^{2+}-ATPases or an anion exchanger (Ahearn, 2010), and the last one can exchange also SO_4^{2-}, Ox^{2-} or even Cl^-. There is a clear impact on genes related to the storage of metals as nickel induces genes that are potentially related to lysosomes. The zinc finger proteins and their related RING finger proteins showed approx. 40 transcripts all being at least 2 fold upregulated. Although these proteins are abundant and their functions are diverse (e.g. DNA recognition or regulation of apoptosis already described above), they are also essential for lipid binding or zinc transport (Laity et al., 2001). Another important group of transcripts is haemocyanin, a protein that transports oxygen in some invertebrate species that contain copper atoms that bind to oxygen (van Holde and Miller, 1995). Haemocyanin can also serve as a transporter for other metals such as zinc as described in the work of Zatta (1984), this fact allied with the upregulated transcripts observed in our study (regulations up to 6 fold) it may be hypothesized that haemocyanin may also transport Ni in a similar way as it transports Zn.

Other transporters that were described to be inhibited by nickel are the epithelial sodium channels (ENaC) responsible for mediating Na^+ transport across high resistance epithelia and participate in the ionic regulation (Sheng et al., 2002). Although these specific channels were not identified in our study, a high number of sodium-solute transporters such as the

sodium-coupled monocarboxylate transporter 2 (SLC5A12) were found to be 3 fold upregulated. Although the function of these second transporters is not associated with Na^+ transport, they play an important role on carbohydrates and lipids transport, reuptake and accumulation. Identically, solute carrier transporters for amino acids and lipids were also 2 - 4.5 fold upregulated in our study (e.g. SLC36A1, SLC16A1, SLC2A8, etc.). Since the mechanism of metal uptake is still poorly unknown, one should not disregard the fact that '*B*' hepatopancreas cells suffer a daily cycle of accumulation and release of carbohydrates and lipids into the midgut lumen as described by Hames and Hopkin (1991). Thus confirming the hypothesis that the accumulation and trafficking of nickel into '*B*' cells may occur through a co-transport and/or moulting processes requiring the accumulation of carbohydrates, lipids and proteins already identified in previous biochemical (Chapter IV) and metabolomic studies (Chapter V), this may also result as part of the detoxification process of terrestrial isopods. To complement this information is also necessary to point out that a different type of sodium-solute transporters appears 2 - 3 fold downregulated which implies a discriminative use of similar pathways that should be further studied.

4.3. Oxidative stress and reactive oxygen species (ROS)

A general toxicity mechanism that has already been identified for metals is oxidative stress and the related ROS production. In the specific case of nickel, biochemical studies involving the enzymatic analysis of oxidative stress (Chapter IV) and nickel impact on metabolites (Chapter V) have already reported that nickel induced oxidative stress to *P. pruinosus,* being one the potential modes of action the inhibition of protein glutathione formation, essential for maintaining the activities of glutathione *S*-transferases (GST), glutathione peroxidase (GPx) or even glutathione reductase (GR). This pattern was confirmed with the observed transcript encoding for GST or GPx that appear regulated up to 4.5 fold, but also other related oxidative stress enzymes appeared as downregulated like the superoxide dismutase Cu-Zn and Mn (-1 to -1.5 fold).

4.4. Neurotoxicity

Mechanisms of neurotoxicity have also been observed in the present study. These mechanisms are mainly related with the inhibition of the acetylcholinesterase (AChE) and have also been depicted in a previous study performed in similar conditions and with the same species (Chapter IV), where a small inhibition of AChE was reported. The transcripts in the present study are related to the encoding of cholinergic and neuro-cholinergic receptors but also other important co-factors (eg. *N*-acetyltransferase necessary for the incorporation of acetyl coenzyme A specific for the formation of acetylcholine, the substrate to be used by AChE) all appearing downregulated up to -3.5 fold. The other neuro-mechanism that also appeared downregulated was the neurotransmitter gamma-aminobutyric acid (GABA) receptors (Kuffler and Edwards, 1958), which has been previously described within a similar pattern in a metabolomic study performed for the same species in the same conditions (Chapter V) thus supporting our findings.

4.5. Reproduction

Impairment on reproduction is expected to occur in *P. pruinosus* exposed to nickel as approx. 20% of the related biological processes were found to be upregulated in both nickel exposure concentrations. Although data is still scarce on the effects of nickel in reproduction pathways, the study performed by Vandenbrouck et al. (2011) using the aquatic organism *Daphnia magna*, showed a downregulation of genes involved in reproduction such as vitellogenin, PFK2-FBPase2 or apolipoprotein d. Also in the study of Evens et al. (2009) the crustacean *Daphnia magna* was exposed to Ni-contaminated food and approx. 33% reduction in reproduction was observed, along with un early time to the first brood but with a reduction in the number of organisms that was consistent also in the following broods for the higher concentrations of exposure (85.6 and 837 µg Ni/g dry food). The study suggested that a variety of mechanisms could be involved that included altered resource allocation or targeted reproductive inhibition. As stated previous, our data shows an impact in the reproductive pathways, but to obtain a more detailed and solid idea of the affected pathways further research should be performed. In fact Ni impacted a high number of transcripts that

encoded cell division that direct and indirectly will affect reproduction and could be seen as one of the mechanisms underneath reproduction impairment, but other transcripts were also found to be impacted. These transcripts include centromere protein I/L (2 fold upregulated) involved in the response of gonadal tissues to follicle-stimulating hormone (Uren et al., 2000) or the hormone vitellogenin (5.5 fold upregulated) a biomarker used for endocrine disruption (Hansen et al., 1998).

5. Conclusions

This work provides the first full body transcriptome of a terrestrial isopod and specifically the species *P. pruinosus*, thus representing an important source of molecular information for these group of organisms. The available data mainly reports to COI used for barcoding but does not represent the overall gene sequences for the major pathways in terrestrial isopods. This transcriptome includes a high similarity to the transcriptome of the aquatic crustacean *Daphnia pulex* and also provides information on the main pathways within these organisms that can be used as a starting point for comparative research.

The second part of the study was based on the RNA-Seq analysis of the impaired pathways induced by nickel exposure. The main results could be categorized into genetic and epigenetic impact, ion trafficking and storage, oxidative stress, neurotoxicity and reproduction. Although similar effects had been observed for other species, the results presented here support and help understanding results presented in previous studies regarding higher organizational levels.

The present work provides the foundations for many molecular researches, but also to understand the metal ion trafficking in terrestrial isopods that in our opinion could greatly help in the future.

Acknowledgment

This study was supported by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia within the research project CLIMAFUN- CLImate Changes and Potencial Impact on Soil FUNctional Ecology (FCOMP-01-0124-FEDER-008656) and through a PhD grant (SFRH/BD/65739/2009) attributed to Nuno G. C. Ferreira by FCT. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to thank the laboratorial support given by Dr. Ceri Moris and Dr. Abel Ferreira.

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CHAPTER VII

Molecular responses of Porcellionides pruinosus (Isopoda) when exposed to dimethoate and nickel.

Molecular responses of *Porcellionides pruinosus* (Isopoda) when exposed to dimethoate and nickel.

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Abstract:

Terrestrial isopods from the species *Porcellionides pruinosus* were exposed to two stressors, the pesticide dimethoate (recommended field appliance dose - 0.4 mg/kg soil, and sublethal concentration - 10 mg /kg soil) and the metal nickel (maximum allowed concentration in the Canadian framework guideline - 50 mg /kg soil; and 250 mg/kg soil). The exposure lasted for 28 days and was followed by a recovery period of 14 days when organisms were changed to clean soil.

Organisms were sampled after 24h, 48h, 96h, 7 days, 14 days, 21 days, 28 days (exposure period), 35 days and 42 days (recovery period). Using real-time PCR, a gene- based analysis was performed to identify the toxic effect within specific pathways. The selected genes belong to pathways related to detoxification, neurotoxicity, cell cycle, DNA replication/repair, apoptosis, oxidative stress, growth and histone acetylation / deacetylation.

The results showed high alteration in the majority of the gene's transcription for both dimethoate and nickel exposures. Also a return to transcriptional levels identical to the control was observed for all genes in organisms pre-exposed to dimethoate (in recovery), but not in all genes from organisms exposed to nickel, suggesting the continuation of effect of this stressor even after its removal.

***Keywords*:** oxidative stress, pesticide, metal, gene expression

1. Introduction

The use of terrestrial isopods in ecotoxicology has already been well established (e.g. Chapter I to VI, (Loureiro et al. 2006); Morgado et al. (2013)), but still little is known about many aspects of its life traits and their molecular responses to stressors. Pesticides are currently used for agricultural proposes, and key organisms like terrestrial isopods can be affected and their functions in the edaphic system impaired. Isopods feed on decayed organic matter and are part of the macrodecomposer community structure and due to their importance in decomposition key processes they have been adopted as a key ecotoxicological species (Jansch et al. 2005; Loureiro et al. 2009; Loureiro et al. 2005; Takeda 1980; Vink et al. 1995). Processes such as aeration, drainage and incorporation of the degraded organic matter are also mediated by isopods that enhance soil quality, nutrient recycling, structure maintenance and fertility (Ferreira et al. 2010; Loureiro et al. 2006; Zimmer 2002; Zimmer et al. 2003). Dimethoate is one of the most extensively used organophosphorous compounds (OP) in agriculture practices, being applied to a great variety of crops. Like the majority of OP, it acts on the enzyme acetylcholinesterase (AChE), inhibiting the degradation of acetylcholine, which produces extensive cholinergic stimulation and neurotoxicity (de Coen and Janssen 2003). The molecular responses of terrestrial isopods to metals are also of great interest, mainly due to their ability to handle high metal amounts, and survive in areas highly contaminated (Donker 1992). The metal nickel (Ni) is a micro-essential metal that although naturally occurring, is present in hot spots at high levels due to anthropogenic activities (Phipps et al. 2002). Knowledge of its effects on soil invertebrates is still very limited, but it is considered a carcinogenic metal, that impacts the phosphate cycle and gene transcription and translation processes (Lee et al. 1995; Pane et al. 2003; Vandenbrouck et al. 2009).

In a previous study by Ferreira et al. (Chapter VI), a global transcriptome analysis of the species Porcellionides pruinosus (Brandt 1983) has been performed and its annotation and mapping provides the foundations to perform ecotoxicological studies using molecular tools. Based on this new available information, this study aimed to understand the impact of two stressors: the metal nickel and the pesticide dimethoate. The major aims were to evaluate the responses of the terrestrial isopod P. pruinosus, when exposed to two concentrations of the

pesticide dimethoate within two relevant concentrations (field recommended appliance dose and a sublethal concentration) and two concentrations of nickel (the maximum allowed limit within the Canadian framework guideline and 5x this concentration). The gene expression through real-time PCR was determined for a 28 day exposure period, followed by a 14 day recover period, within specific target pathways (e.g. cell division, DNA repair, detoxification, oxidative stress and growth). The results obtained will help to understand the processes that isopods undergo when exposed to these stressors in time and how the transcription mechanisms will work for the organism to cope with the stress, and the effort to reach a new homeostasis status. It will also help understanding the physiological mechanisms of this species underlying the toxicity of a model pesticide and metal.

2. Materials and methods

2.1. Test Organism and Culture Procedure

Organisms belonging to the species *Porcellionides pruinosus* Brandt (1833), maintained for several generations in laboratory cultures and previously collected from a horse manure heap were used as test organisms. They were maintained at 20 ± 1°C, with a 16:8 h (light:dark) photoperiod and fed *ad libitum* with alder leaves (*Alnus glutinosa*). Maintenance of cultures was performed in a week basis and whenever necessary water was sprayed and food provided. For the experiment only adult organisms (15-25 mg wet weight) were used and no distinction between genders was made, although pregnant females, organisms with abnormalities, or moulting characteristics were excluded.

2.2. Soil Spiking

The metal nickel and the pesticide dimethoate were spiked in LUFA 2.2 soil. Nickel was spiked as nickel (II) sulfate hexahydrate ($NiSO_4.6H_2O$) in two concentrations: 50mg and 250mg Ni/kg soil, with a final moisture content equivalent to 50% of the soil water holding

capacity (WHC). The concentration of 50 mg Ni/kg soil, represents the maximum concentration allowed in the Canadian framework guideline (CBP, 2010), and both concentrations can be found in the environment (Figueira et al., 2002). As for the pesticide, dimethoate was spiked with 0.4 mg and 10 mg dimethoate/kg soil, also with a final moisture content equivalent to 50% WHC. The concentration of 0.4 mg dimethoate/kg soil represents the recommended field dose after dimethoate application and the 10 mg dimethoate /kg soil was used as a level below EC50 value found for the isopod *Porcellio scaber* (Ferreira et al., 2015; Fischer et al., 1997).

2.3. Experimental procedure

Exposures were performed in plastic boxes (26 length x 18 width x 7.5 height cm), with approx. 2cm height of natural LUFA 2.2 soil layer (Speyer, Germany) and 32 isopods (per box). Culture organisms were weighed (15-25mg) and placed in each test-box. Alder leaf disks (Ø 10 mm, ± 20 mg) were supplied initially in small quantities as food, and whenever necessary, in order to prevent organisms remaining on the top and avoiding exposure. Organisms were exposed in similar conditions to the ones in culture, with a 16:8 h (light:dark) photoperiod, at 20ºC± 1°C. A total of three organisms were collected for each treatment and time. One organism was collected from each box/replicate at the following sampling times: 0h, 24h, 48h, 96h, 7 days, 14 days, 21 days, 28 days (exposure period) and 35 days, 42 days (recovery period). In the results section, 35 and 42 days of test duration will be denominated as 7 and 14 days of recovery.

2.4. RNA extraction

Total RNA was extracted from each individual organism using Trizol® Reagent (Ambion) followed by a column purification step with RNAeasy Mini Kit® (Qiagen) and stored at -80 °C. Prior to freezing, an analysis of the A260/280 and A260/230 ratios of all the RNAs was

measured using Nanodrop 2000c spectrophotometer (Nanodrop Technologies, USA). Prior to the following steps, some samples were randomly selected and analysed for their integrity.

2.5. Quantitative real time PCR (RT-qPCR)

A total of 500 ng RNA from each sample was reverse-transcribed to cDNA using QuantiTect® Reverse Transcription Kit (Qiagen) according to manufacturer's instructions which included a genomic DNA elimination step. The synthesized cDNA samples were stored at -20 °C until they were used in RT-qPCR assays. The primer design was performed using Roche's Universal ProbeLibrary (https://www.roche-applied-science.com/) after a melting curve analysis to select the optimum primers (Table 1.1). RT-qPCR was performed using a Stratagene Mx3000p® (Agilent, UK) in a 20 µl reaction mix containing 5 µl cDNA sample, 10 µl 2x qPCR SyGreen 1-Step Lo-ROX mix (PCR Biosystems Ltd, London), 2.5 µl each primer (4 µM). The RT-qPCR cycling conditions followed several steps, starting with a denaturation phase (2' at 95ºC), then amplification phase of 40 cycles (30'' at 95ºC, 1' at 60ºC) and a final melting phase (1' at 95ºC, 30'' at 55ºC, 30'' at 95ºC). A reaction without DNA was used as the no template control, and another reaction using cDNA from a culture organism was used as a "calibrator cDNA" for normalisation between microplates.

The gene expression values were normalized using the housekeeping gene translation elongation factor 2 (EF2), and this selection was performed after the previous analysis of four potential housekeeping genes: α-tubulin, translation elongation factor 2, actin and 60S ribosomal protein using NormFinder (Andersen et al., 2004). The stability values found after independent analysis of Ni and dimethoate exposures or both at the same time, gave always consistent results: EF2 < 60S ribosomal protein < actin < α-tubulin. Relative mRNA expression levels were calculated using the $2^{-\Delta CT}$ method described by Livak and Schmittgen (2001).

Table 7.1 Genes used for RT-qPCR, with their respective acronym, respective pathway, sense and antisense sequence.

Gene Name	Acronym	Pathway	Sense (5'-3')	Antisense (3'-5')
Topoisomerase (DNA) II Alpha 170kDa – TOP2A	TOP2A	Cell Cycle Checkpoint, DNA Repair	AATAATGGAAATTGTATCCCCTTG	TCACCAGTTTTACAGTCTTTTTCG
Transformation/Transcription Domain-Associated Protein -TRRAP	TRRAP	DNA Repair	CACTTTCCAGATCATAAAGCACCT	AAGTGCAATGAGTGCTACATCAA
Sirtuin 1 – SIRT 1	SIRT1	Apoptosis	TTTTCCCAAGTTCTTATAATCACCA	AAGAATCAGAGATTAAGCAATTACCAT
Vitellogenin	VIT	Reproduction	ACCGGACTTTCGTCTTGTGT	CAAAGTTCAAGAACCAGGGAGA
Acetylcholinesterase	AChE	Neurotoxicity	TGCCTTTTGGAAAAAGTTCCT	CAGGCCCCTTTGAGTGTTTA
Glutathione Peroxidase 3	GPx	ROS	TGATAAGTCTCGTTGGTCATGC	CAGGGGAAAGGTTTTTGTGA
L-Lactate dehydrogenase	LDH	Energy usage	TCTTACATATGTGGCGTGGAGA	CCAGACCCTATGACTCGGTTT
Glutathione *S*-transferases 1, isoform D	GST	ROS	CACGATCCTGAGCTGACTGA	GCATCAAAGCCCTTGCTTA
Hemocyanin B chain	HCya	Metal Binding	CTGAATTAAAGGAGGCATCTGAG	ACAGCTTCTCCTCCGTCAGT
Superoxide Dismutase [Cu-Zn]	SOD	ROS	AGAGCCTCAAAACTGGCAAC	TTGACACTCTCAATGGTACAACAA
Cytochrome P450 71D94	P450	Detoxification	GCACAACTATACGGTTTTCACG	TGTGTACATAGAAGCCATTACAACG
Chitinase 3	CHIT	Growth	ACTTTGGAACCGAAGGAGAAC	TCGCATTTTGTGTGACGTTT
Tubulin alpha-1 chain	Tub	Housekeeping Gene	CCTCTTCCTCCTTTGACATGG	ACAGGCTCGGAGGGAGAC
Translation elongation factor 2	EF2	Housekeeping Gene	CAAATACACATTGTGGGAATGC	TTCGGTTTTACTGCCGATTT
Actin, cytoplasmic 1	Act	Housekeeping Gene	CCAAAAATGATGGTTGGAAGA	TTACCATCGGTAACGAGAGATTC
60S ribosomal protein	Rib60S	Housekeeping Gene	TCTGACTTTTCAAATTTTTGGGTA	CAACATGGACAAAGTACCAAGC

2.6. Data analysis

The obtained data was plotted into a heat map using the GENE-E software (http://www.broadinstitute.org/cancer/software/GENE-E/index.html). The software was also used to perform a hierarchical cluster analysis of genes within each exposure concentration of dimethoate and nickel. The hierarchical cluster analysis was performed using a Pearson Correlation Coefficient with Single Linkage. In order to infer statistical differences between control and exposure concentrations, one-way analysis of variance (ANOVA) or a Student's test was performed. When data did not show a normal distribution or normal variance, the non-parametric test Kruskal-Wallis One Way Analysis of Variance on Ranks was used followed by a Dunn's comparison test in order to discriminate statistically different treatments from the control. For two treatments comparison was used a Mann-Whitney Rank Sum Test (SPSS, 1999).

3. Results

During exposure, no mortality was observed for the control or the lower exposure concentration of nickel (50 mg/kg soil) and 3 organisms were found dead for the higher concentration (250 mg/kg soil). For the dimethoate exposure, at the end of the experiment, the number of dead organisms was 6 and 11 respectively for 0.4 mg/kg soil and 10 mg/kg soil (without considering the organisms sampled from a total of 32 organisms).

The ANOVA performed to distinguish control and treatments showed no significant difference between any treatment and its corresponding control for either dimethoate or nickel's exposure (Table 1SD and Table 2SD).

The results from the cluster analysis are presented in Fig. 1 from organisms exposed to dimethoate (A- 0.4 mg/kg soil, B- 10mg/kg soil) and organisms exposed to nickel (C- 50 mg/kg soil, D- 250mg/kg soil). For dimethoate gene transcriptional differences ranged approx. between -5.5 and 7.5 fold, and for nickel the range was approx. between -6.8 and 8.4 fold when compared to the control.

For the exposure to the lower concentration of dimethoate (Fig. 1A), it was possible to observe the gene TOP2A separated from all the other genes. Also the genes SOD, GST, GPx and SIRT, mostly associated with detoxification processes and oxidative stress handling, appeared also clustered. As for the cluster composed by the genes CHIT and CYP450, they showed the highest similarity and can be related to processes of detoxification and moulting. Finally the group formed by HCya, AChE, LDH, TRRAP and VIT all belonging to different processes form another cluster. When the exposure to the higher concentration of dimethoate was analysed (Fig. 1B) CHIT and CYP450 continue to show the higher similarity, with VIT associated with these two genes but with lower similarity. The other branch of the cluster showed again a cluster with the detoxification enzymes SOD and GST, and another group formed by genes involved in different processes (TOP2A, TRRAP, AChE, HCya, SIRT, LDH and GPx). From these SIRT and HCya appeared the most dissimilar from the others, whereas TOP2A, TRRAP and AChE, almost all involved in epigenetic traits, appeared together. A cluster of high similarity was also observed for LDH and GPx.

For the exposure to the lower concentration of nickel (Fig. 1C), it was possible to observe GST in a branch separated from all the other genes, from which was again the most similar transcriptional genes, forming a cluster with other detoxification genes – GPx and SOD. It was also possible to observe a cluster of AChE and HCya and another cluster between TOP2A and TRRAP. For the exposure to the higher concentration of nickel (Fig. 1D), CHIT, CYP450 and VIT appeared in a separated branch of the cluster. In the exposure to the lower concentration LDH appeared mostly separated from the others and AChE and HCya clustered together but this time in a major group that involved the detoxification and oxidative stress handling genes (SOD, GST, GPx), along with SIRT and TRRAP that can be related to DNA damage.

The transcriptional changes in the analysed genes for organisms exposed to dimethoate and nickel are shown in Fig. 2 and Fig 3 respectively. For organisms exposed to dimethoate, major changes between the two treatments are observable during the period between 21 and 28 days (AChE); 24-48h and 14-21 days (TOP2A); 21 days (TRRAP); all the exposure period except for the first 48h (GST); 48h, 21 and 28 days (HCya); 28 days (GPx); 21 days (LDH) and 48h (VIT). The genes SIRT, CHIT, P450 and SOD show similar transcriptional patterns. As for organisms exposed to nickel these changes are observed for all the exposure

and recovery period except the first 48h (TOP2A); 7-14 days (VIT); 48h to 21 days (GPx); 28 and 42 days (HCya); 48h (GST); 7 to 42 days, except 28 days (CHIT and P450). The genes SOD, LDH, SIRT, TRRAP and AChE show similar transcriptional patterns.

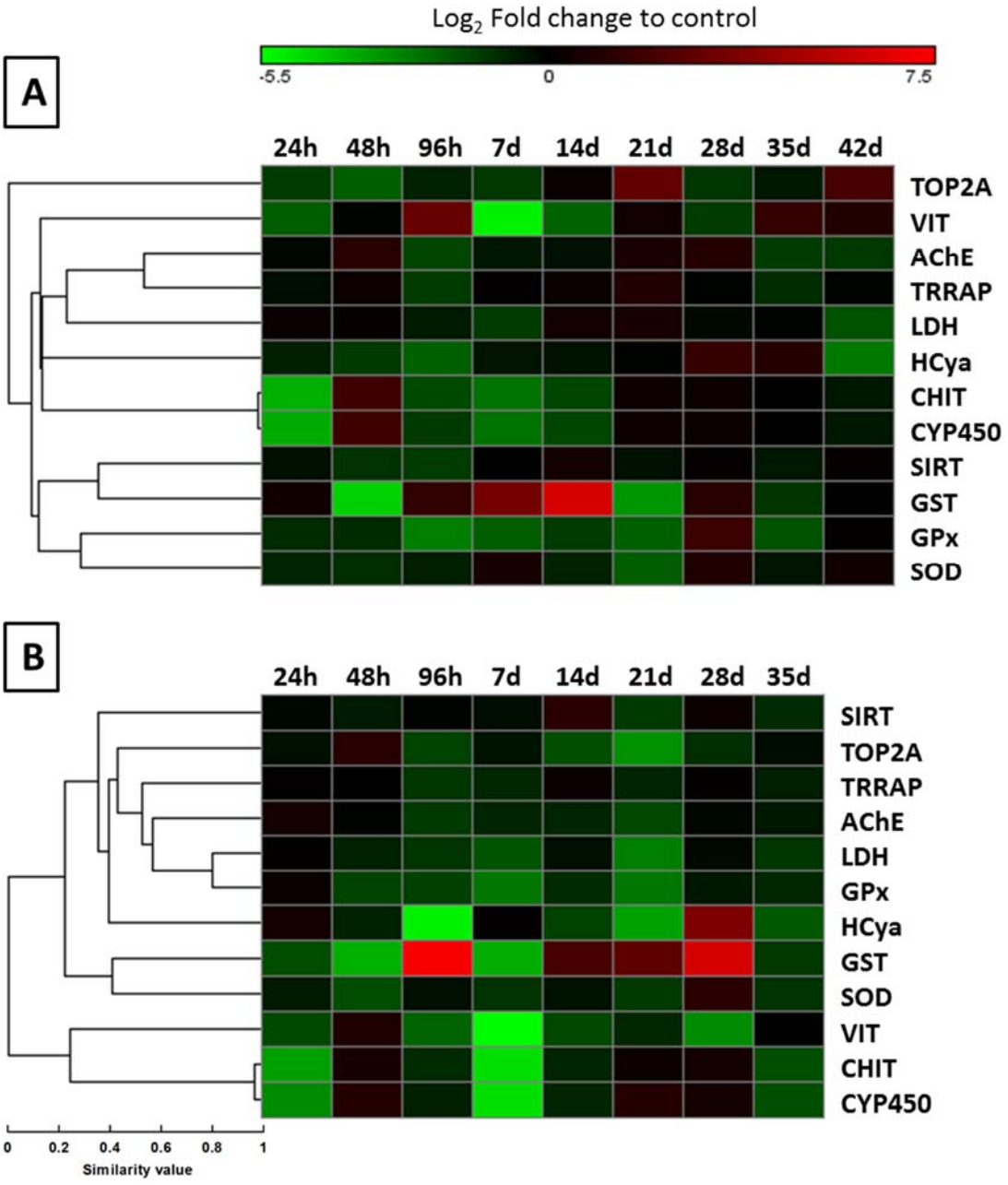

Fig. 7.1 Heat map showing cluster of gene's mean log_2 transcription differences between organisms from the species *Porcellionides pruinosus* in control and exposed to (A) 0.4 mg dimethoate/kg soil (B) 10 mg dimethoate/kg soil (C) 50 mg Ni/kg soil and (D) 250 mg Ni/kg soil after 24h, 48h. 96h, 7 days, 14 days, 21 days, 28 days (exposure period), 35 days and 42 days (recovery period).

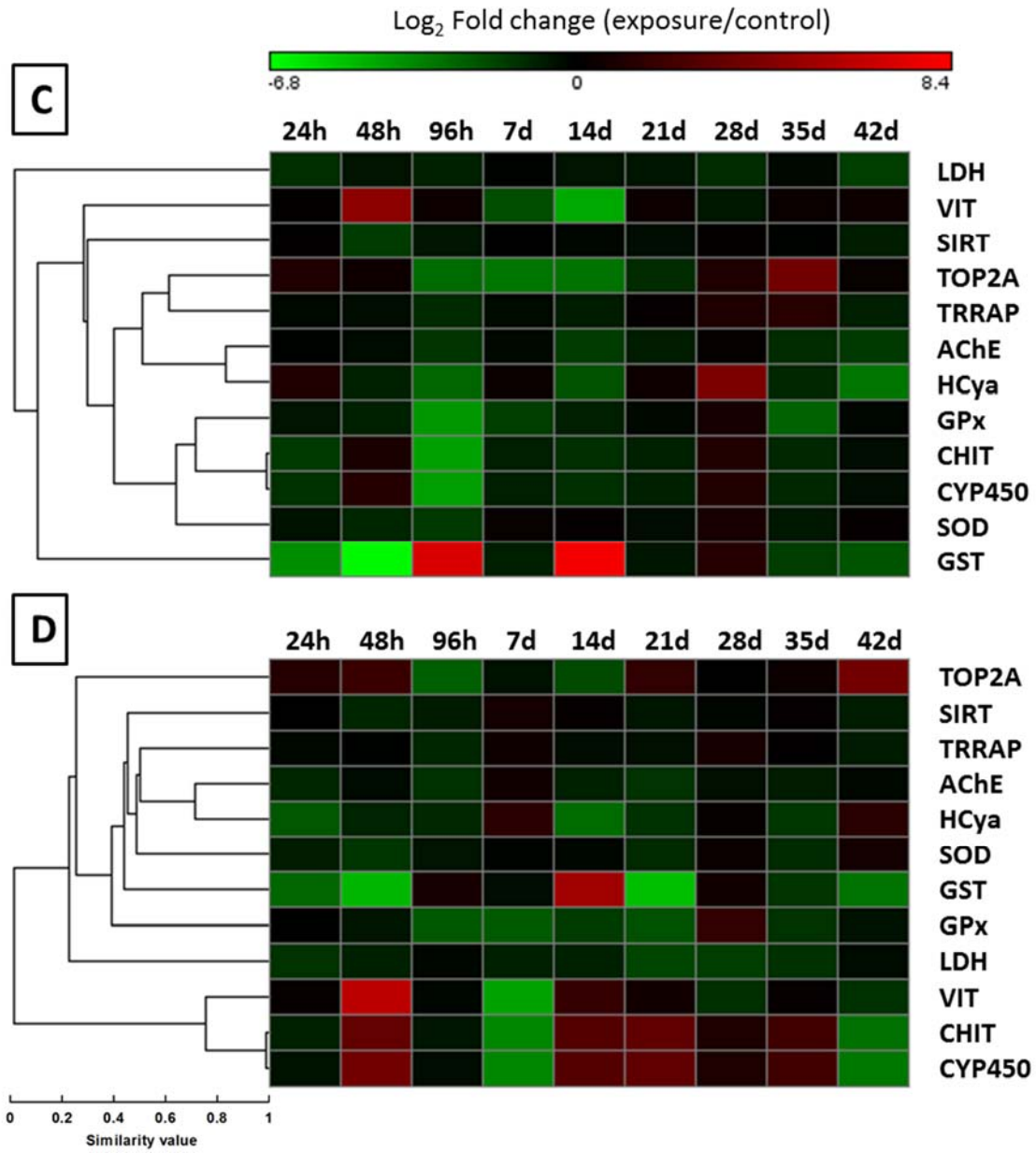

Fig. 7.1 (cont.) Heat map showing cluster of gene's mean log_2 transcription differences between organisms from the species *Porcellionides pruinosus* in control and exposed to (A) 0.4 mg dimethoate/kg soil (B) 10 mg dimethoate/kg soil (C) 50 mg Ni/kg soil and (D) 250 mg Ni/kg soil after 24h, 48h. 96h, 7 days, 14 days, 21 days, 28 days (exposure period), 35 days and 42 days (recovery period).

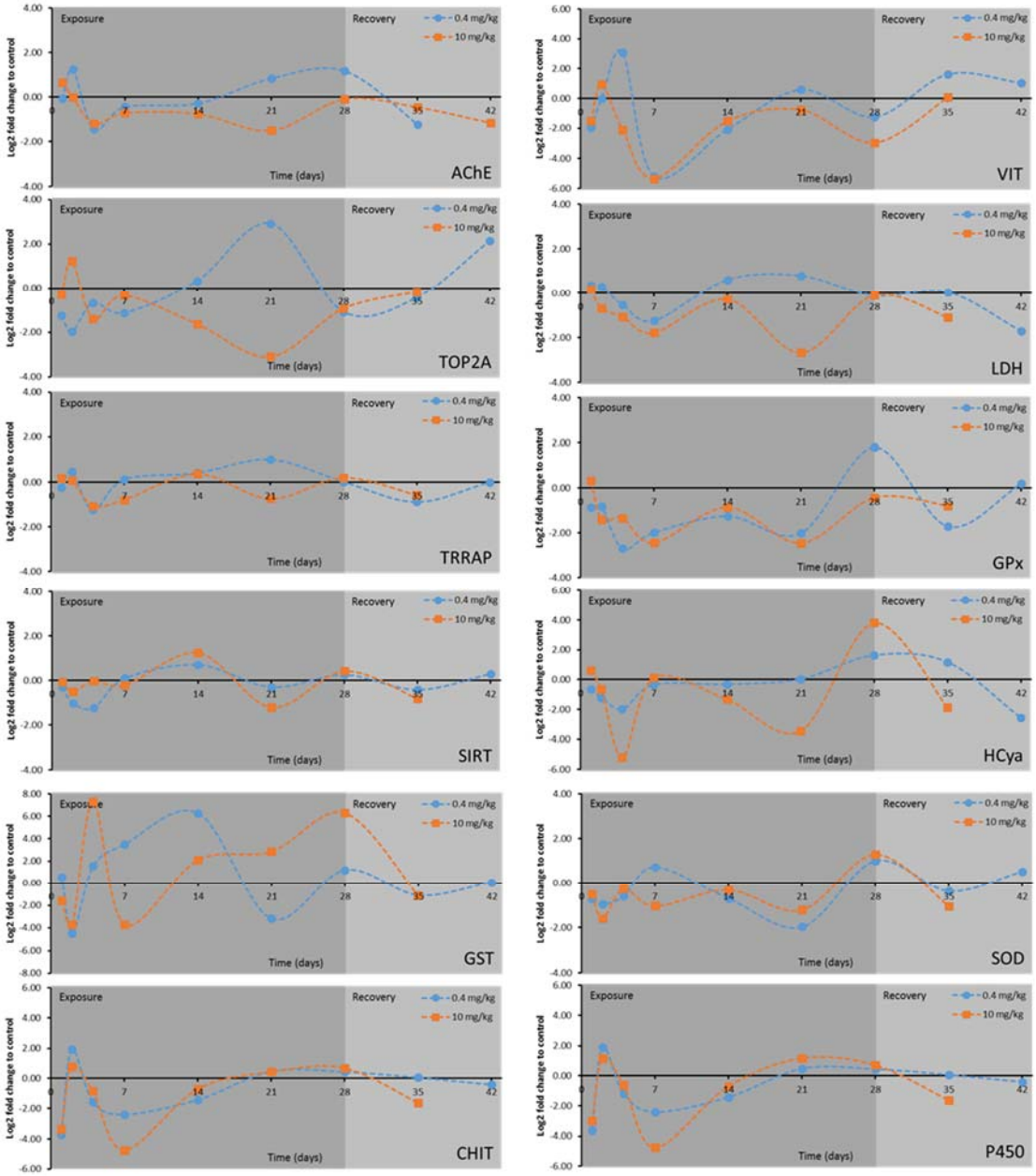

Fig. 7.2 Mean transcriptional differences between organisms from the species *Porcellionides pruinosus* in control and exposed to 0.4 mg dimethoate/kg soil and 10 mg dimethoate/kg after 24h, 48h. 96h, 7 days, 14 days, 21 days, 28 days (exposure period), 35 days and 42 days (recovery period).

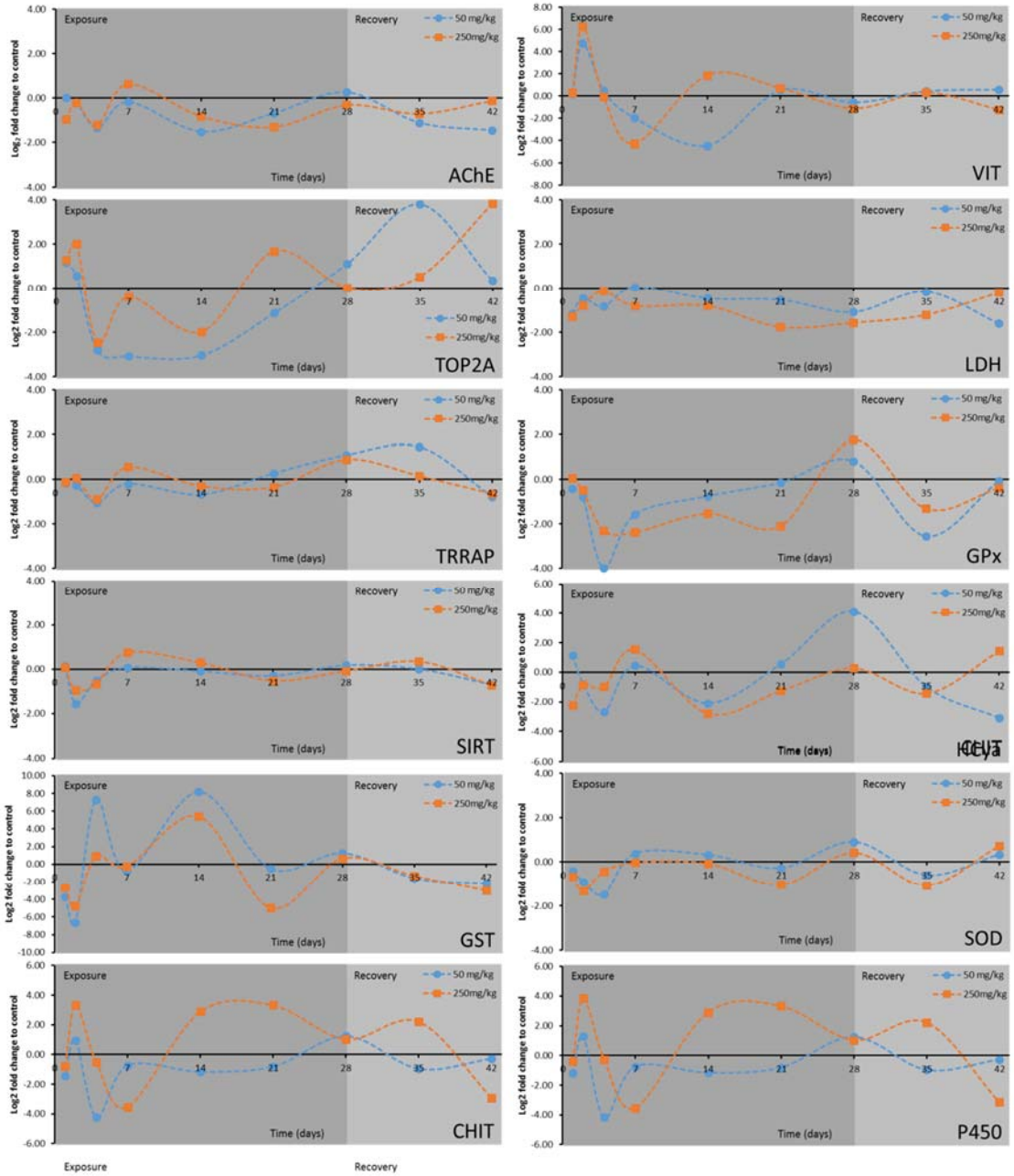

Fig. 7.3 Mean transcriptional differences between organisms from the species *Porcellionides pruinosus* in control and exposed to 50 mg nickel/kg soil and 250 mg nickel/kg after 24h, 48h. 96h, 7 days, 14 days, 21 days, 28 days (exposure period), 35 days and 42 days (recovery period).

4. Discussion

The present work pioneers the exploration of molecular tools to evaluate toxicological effects of a model pesticide and a model metal within the order Isopoda. Four genes were considered as candidates as the housekeeping gene (HKG) to be used in this work, based in literature (e.g. Stürzenbaum and Kille (2001); Thellin et al. (1999); Vandesompele et al. (2002)) and a previous Next Generation Sequencing RNA-Seq analysis - NGS (please see Chapter VI). However, the time course analysis suggests a significant variation within the compensation phase, which may lead to an undue influence of the HKG in the measurements of our target molecular biomarkers. Furthermore, although not perceptible in other works that are based in higher organizational levels (e.g. Chapter IV and V), the first 4 to 7 days of exposure show significant dynamic changes in gene expression consistent with the compensatory mechanisms of the organism. Another important fact to take into consideration when comparing the NGS RNA-SEq analysis and the RT-qPCR data is the exposure regimes. In fact, although a similar ratio of organisms per area was maintained in both exposures, the dissimilar number of cohort (6 times higher in this study) may pose an additional stress factor. The resulting variation and the high dynamic responses found within the compensation profile, makes it challenging to align the precise response in the two different molecular experiments. Nevertheless, this does not compromise or excludes the application of RT-qPCR or NGS RNA-Seq analysis to underlie the molecular mechanisms of action from these compounds.

The overall analysis of gene transcription, although as expected very different between the two model stressors, show very similar patterns between the lower and higher concentration. The majority of sampling points show an increase in response as the exposure concentration increases. Also, during the recovery period, the majority of the genes analysed in this study, show levels very similar to control after 14 days. Nonetheless, it is important to note that when a recovery is not observed it can be reported almost always to the highest concentration of exposure.

4.1. Exposure to dimethoate

The analysis of the two exposure concentrations of dimethoate brought different results of the gene's transcription clustering; nevertheless some specific clusters of genes are maintained in both analyses. One of these clusters is composed by the genes CHIT and CYP450 that belong to different pathways. These genes show a high fluctuation within 7 days of exposure, after which they tend to return to control values. The gene CHIT encodes a protein/enzyme that is responsible for the degradation of the chitin so the organisms can replace the older carapace and complete a moulting process (Mira, 2000; Steel, 1993) and CYP450 is involved in detoxification processes in the phase I of the biotransformation of endogenous and exogenous compounds (Ren et al., 2014; Rewitz et al., 2003; Ricciardi et al., 2010). Although apparently they may be completely unrelated, the biotransformation of dimethoate may be complementary to the moult process through which the organism will excrete the metabolites resulting from its degradation. These interconnected processes are here presented for the first time, although being suggested in several previous studies (Chapter III and V), in fact moulting processes have been more widely associated with metal exposure. For example, in the study of Drobne and Štrus (1996)), an increase in moulting frequency was related to an increase in zinc contaminated food.

Another cluster is formed by the detoxification and oxidative stress related genes (SOD, GST and GPx) along with the gene SIRT that can also be observed for the lowest exposure concentration. This pattern is not visible for the highest concentration, although a cluster of the detoxification genes GST and SOD continues to be present. The enzyme GST presents the higher variation within time as it ranges from -4 to 8 fold regulation. It is also noteworthy to note that this gene shows the most differences between organisms exposed to the lower (0.4 mg/kg soil) and higher concentration (10 mg/kg soil). Independently, this high variation is not observed for GST activity evaluated in a previous study (Chapter III) where organism from the species *P. pruinosus* were exposed in the same conditions to dimethoate and do not show identical patterns of induction/inhibition of activity. Although caution should be taken when comparing both studies, as we are analysing only GST-1 isoform, it is possible to observe a opposite pattern between GST transcription and GST activity, for example after 24h of exposure GST activity is induced for both concentrations (approx. 25%) in contrast

with GST transcription that is -4 fold regulated. The biological significance of these results is difficult to ascertain, nevertheless the high transcriptional variance may be indicative of a high influence of this isoform in dimethoate detoxification and further research should be performed. As presented in previous studies (Chapter III and V), organisms also belonging to the species *P. pruinosus*, when exposed to identical concentrations of dimethoate also showed an impairment in detoxification (GST) and oxidative stress handling enzymes (CAT an GPx) along with cell damage (LPO rate). The same was observed in the study of Wilczek et al. (2013) where an increase of GST activity was observed for females of the species *Xerolycosa nemoralis* exposed to dimethoate. As for SOD although some variation between concentrations could be observed at the 7th day of exposure, the overall transcription is very similar although differing in intensity. Also a transcriptional variation is observed between -2 and 2 fold regulation which is in accordance with previous studies performed with spiders from the species *X. nemoralis* that when exposed to dimethoate showed an increase in SOD activity (Wilczek et al., 2013) or the study of Novais et al. (2012) that showed an increase in SOD transcription in approx. 1 fold for *Enchytraeus albidus* exposed to their EC50 concentration of dimethoate (2 mg/kg soil). The third gene that falls within similar patterns for the lower concentration (SIRT) is a very distinct protein than the previous enzymes that is associated with different traits that range from coordinating cell cycle, responses to DNA damage or apoptosis to epigenetic traits such as deacetylation of histones and alterations in the methylation of histones or DNA (Voelter and Mahlknecht, 2006; Zschoernig and Mahlknecht, 2008). The clustering of SIRT with GST, GPx and SOD when exposed to lower concentrations of dimethoate and its separation at higher concentrations may be indicative that its major function is related to DNA damage and apoptosis since in the previous study (Chapter III) organisms from the species *P. pruinosus* exposed to the same concentrations of dimethoate showed high lipid peroxidation rates (LPO) and high mortality after 14 days of exposure to the higher dose of dimethoate (10 mg/kg soil). Nevertheless, one should not disregard SIRT role in epigenetics but at the present time, no studies have been performed to investigate the role of SIRT in this context.

As stated previously, the gene GPx clusters along with GST, SIRT and SOD at lower concentrations of exposure but changes into a separate cluster with LDH when it reports to higher concentrations of exposure. GPx is an antioxidant enzyme responsible for protecting

cells from oxidative damage by the reduction of hydrogen peroxide using glutathione reduced as co-factor (Diesseroth and Dounce, 1970). The grouping of this gene along with the other antioxidant genes (GST and SOD) is in accordance with its role and is also supported by the study (Chapter III) that shows the encoded enzyme activity to be impacted by dimethoate in the same species (*P. pruinosus*) over the same concentrations of dimethoate, thus also supporting the idea that dimethoate generates oxidative stress. Although the cluster for the higher concentration of exposure presents itself differently from the lower one, its role may be indicative of a shift in the regulation process of these organisms. In fact it seems that the conversion of pyruvate into lactate through the enzyme LDH, or instead the transformation of pyruvate into acetyl Co-A may shift the energy available for the activity of GPx. In the study of Crane et al. (1982) showed that acetyl Co-A interacts with glutathione forming a complex that will decrease GPx activity by 5%, that depends on glutathione reduced to function. This decrease could lead to the organism increasing GPx gene transcription as a feedback response to raise GPx activity.

The gene haemocyanin (HCya), a protein that transports oxygen in some invertebrate species, contains copper atoms that bind to oxygen and that can also serve as a transporter for metals such as zinc (van Holde and Miller, 1995; Zatta, 1984). It appears in both the exposure concentration investigated. Besides these roles, HCya is also involved in moult processes as described by Sellos et al. (1997), in a study that found a 3 fold increase during the premoult stages of the species *Penaeus vannamei*. In fact, although its large transcription that ranges from -2 to 2 fold and -6 to 4 fold regulation respectively for 0.4 mg and 10 mg dimethoate/kg soil the changes observed might be attributed to moult. This is suggested by other studies (Chapter III and V) that found evidence of an impact in moult processes, that measured biochemical biomarkers, energy reserves content and metabolites levels in the same species (P. *pruinosus*) over the same concentration range.

The vitellogenin (VIT) gene although being present as a marker for endocrine disruption (Hansen et al., 1998) may also be involved in the moult process as described in the review of Meusy (1980). In the study of Okumura and Aida (2000), changes in vitellogenin levels were also observed for the species *Macrobrachium rosenbergii*, but although a relationship between moult and vitellogenin levels has been determined, the mechanism behind its exact relationship has not yet been discovered. In our study, the transcription of the gene VIT

undergoes a major variation between 4 and -6 fold regulation within the first 7 days of exposure, returning to more stable levels afterwards. This period of time is in agreement with moult events observed in this and in prior studies (Chapter III and V).

For the lower concentration of exposure a cluster between AChE and TRRAP can be observed, although when reporting to the higher concentration of exposure these two genes appear separated. The gene AChE encodes for the enzyme acetylcholinesterase which is responsible for the hydrolysis and inhibition of the neurotransmitter acetylcholine, producing choline and an acetate group. AChE is the main target of the pesticide dimethoate that acts to inhibit it (Purves et al., 2008). As for TRRAP, it encodes a protein involved chromatin complexes with histone acetyltransferase activity thus serving as a specific tag for epigenetic transcription activation (Sawan et al., 2008). Although the reason of their clustering at lower concentrations of exposure cannot be directly explained, a possible explanation may be due to the epigenetic alterations in which TRRAP may act that will then control also the transcription of the gene AChE although this assumption steel needs to be proved. In fact, AChE transcription should be expected to be upregulated throughout the time of exposure in order to deal with the inhibition of AChE activity caused by dimethoate as a mechanism for the organisms to return to its homeostasis status. Nevertheless the opposite is observed and this downregulation might be caused by epigenetic traits.

As for TOP2A gene, both the exposure concentrations show its transcription as isolated. This gene encodes DNA topoisomerases responsible for overcoming topological problems in genomic DNA that result from DNA replication, transcription and repair (Wang, 2002). The major transcription differences observed for this gene between the exposure concentrations are at 48h and 21 days of exposure. At 48h at the lower concentration an induction of approx. 2 fold is observed whereas for the higher concentration a decrease of approx. -2 fold is observed. At 21 days the scenario is the opposite but the fold change is approx. -3 and 3 fold, being the gene that shows more differences between lower and higher exposure concentrations. These results may be related to the negative effects of oxidative stress induced by dimethoate. For example, in a similar study (Chapter III) the increased LPO rates (21 days of exposure) contrast with the TOP2A transcription thus showing a protective effect. This is also supported by the study of Grosicka-Maciąg et al. (2012) that also shows

a relationship between the fungicide zineb and dimethoate oxidative stress damage and the alterations in topoisomerases.

It is also noteworthy to highlight the fact that all the analysed genes after the organisms being changed to clean soil, show a recovery pattern and their transcription levels returned to the control levels.

4.2. Exposure to nickel

Similarly to dimethoate nickel showed different transcription clustering between the exposure concentrations, but a few of the clusters were maintained such as the CHIT and CYP450 cluster. The fact this cluster was also observed for the dimethoate exposure further supports the idea that this species undergoes a detoxification process after exposure that leads to moult and excretion of the xenobiotic whether it is a pesticide or a metal. At the lower exposure concentration, these genes report also very close to GPx and SOD transcription. This is supported by previous studies (Chapter IV and V) where organisms from the same species were exposed to the same concentrations of nickel and this metal was found to induce oxidative stress. The generated oxidative stress and the respective handling enzymes should then show a similar pattern as observed. As for the higher concentration the cluster of CHIT and CYP450 shows a closer affinity to VIT and not to GPx or SOD. This result may demonstrate a concentration dependent role of VIT in moult processes as suggested for the dimethoate exposure, as at lower concentrations VIT may play a less significant role.

Another cluster of genes that is identical to both exposure concentrations is formed by AChE and HCya. The results obtained for HCya would be expected as this protein transports oxygen in some invertebrate species and can also serve as a transporter for metals (van Holde and Miller, 1995; Zatta, 1984) as well as being involved in moult processes (Sellos et al., 1997). The first 7 days of exposure show a pattern that can be identified with dimethoate pattern but also with energy reserves content (Chapter IV) and metabolites levels (Chapter V) found for this species and that can be related to moult. In the same way, the pattern

observed during the rest of the exposure period may be related to its role in the trafficking of metals, which was also observed for a RNA-Seq analysis performed for the same species and the same concentrations of nickel (Chapter VI). As for AChE, nickel has been described to impact neurotransmission and in a previous study (Chapter VI) that used the same species and the same exposure conditions showed an impact in this gene and related co-factors that act in the same neurotransmission pathway. This study is also complementary with another study (Chapter IV) also performed with the same species in the same conditions and concentrations of nickel that shows inhibition of AChE enzyme activity. Although no relation can be obtained at this moment for the clustering of AChE and HCya, future studies focuses in metal trafficking within terrestrial isopods may bring new insights.

Another cluster that is evidenced at the lower concentration of exposure is TOP2A and TRRAP. Although the transcriptional changes in TRRAP appear not be as intense as in TOP2A, the clustering of these two genes may suggest a relation between chromatin changes, DNA repair and apoptosis systems. As reported in a previous study (Chapter VI) the species *P. pruinosus* when exposed to the same concentrations of nickel showed an impact epigenetic factors that included chromatin and histone modifications, but also in DNA repair and/or pro-apoptosis systems that are impaired and may be the cause of its carcinogenic toxicity. These toxicological effects of nickel have also been reported in several other studies that report base mis-incorporation into new synthetized oligonucleotides, DNA repair inhibition or even cell cycle arrest (e.g. Ahamed et al. (2011); Sirover and Loeb (1976)). As for the higher concentration of exposure TOP2A appears separated from all the other genes and TRRAP appear close to the cluster of AChE and HCyA. This may denote for one side that the effect of nickel on DNA replication and repair may have a higher impact (Showed by TOP2A) than at epigenetic level (showed by TRRAP), which can also be seen by the time transcriptional variations showed between genes and between within each gene between exposure concentrations.

An important gene transcription to highlight is the GST. This gene appears isolated in both exposure concentrations, although at the higher concentration one should highlight that other oxidative stress handling genes SOD and GPx appear next to it. As presented in several previous studies the encoded enzyme resulting from this gene appears impacted, this can be seen for example for the study (Chapter IV) performed with the same species and same

conditions of exposure to nickel that shows GST activity for a similar exposure and recovery period, or the RNA-Seq analysis (Chapter VI) that shows an impact in GST gene. All of these studies highlight the protective role of GST in handling oxidative stress and the -8 to 8 fold regulation indicates a strong role of the isoform presented in this study (GST- isoform 1) for this specific terrestrial isopod species as it is highly regulated in both dimethoate and nickel exposure. Regarding the higher concentration of exposure one should also take in consideration that GPx and SOD clustering near GST may be due to their role also in handling oxidative stress. Also GPx activity will also be directly interconnected with GST as glutathione reduced will be necessary for both enzymes to work. Also, a metabolomic study (Chapter IV) that used the same species and exposed organisms to the same concentrations also showed an impairment in the formation of glutathione which can be considered a cause for the high transcription of glutathione related genes since at a higher organizational level the proteins/enzymes are not being formed.

The last two genes evaluated for nickel LDH and SIRT both present themselves isolated for both exposure concentrations. The LDH gene appear within the sampling times always being downregulated and although it encoded enzyme activity has not been determine in previous studies and so a relation between activity and transcription cannot be determined, the most plausible explanation may be that nickel in opposition to dimethoate may not be using the anaerobic pathway that converts pyruvate into lactate and vice versa, but the aerobic pathways that converts pyruvate into acetyl Co-A. As for SIRT although its role in epigenetic modifications have also been reported in a previous study (Chapter V) that using the same species evaluated nickel toxicity and showed an impact in this gene. Here is showed to have little to almost no impact in nickel toxicity, which could indicate that TRRAP have a more active role as an epigenetic factor than SIRT.

Unlike dimethoate, after the organisms had been place on clean soil and started their recovery, most genes showed not to return to control transcriptional levels. An example of this is TOP2A, which indicates that these organisms still evidence a strong toxicological effects of nickel even after 14 days of recovery.

5. Conclusions

The effects of a model pesticide (dimethoate) and a model metal (nickel) were evaluated through a transcriptional analysis throughout an exposure period of 18 days, followed by a recovery of 14 days. The results obtained for dimethoate exposure, showed very similar profiles between the lower and higher concentrations of exposure, indicating oxidative stress, neurotransmission impairment and even some epigenetic factors related to DNA repair and/or pro-apoptosis along with chromatin changes and cell cycle arrest. Similar evidences were observed for nickel exposure, although the exposure to the different concentrations showed distinctive patterns. Other important highlight from the study was the fact that organisms exposed to dimethoate when transferred to clean soil and left for recovery for 14 days returned to a near control transcription, whereas the organisms exposed to nickel did not show similar control transcription. This may suggest that nickel can continue to impact organisms even after the stressor has been removed.

Acknowledgment

This study was supported by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia within the research project CLIMAFUN- CLImate Changes and Potencial Impact on Soil FUNctional Ecology (FCOMP-01-0124-FEDER-008656) and through a PhD grant (SFRH/BD/65739/2009) attributed to Nuno G. C. Ferreira by FCT. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to thank the technical support provided by Dr. Abel Ferreira.

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CHAPTER VII

General conclusions

1. General conclusions

The important role of terrestrial isopods in ecosystems and their use as test organisms in ecotoxicology has already been widely demonstrated and investigated (e.g. Calhôa et al. 2012; Drobne 1997; Ferreira et al. 2010; Loureiro et al. 2006; Morgado et al. 2013). Nevertheless techniques involving the evaluation of sub-individual parameters, and more specifically at a proteomic, metabolomic, transcriptomic and genomic levels, are still not widely used. In this study, the approach chosen was performed in order to fill this gap and present the toxicological effects of a model pesticide (dimethoate) and a model metal (nickel).

Within the following paragraphs, the information obtained in this study will be compiled into an adverse outcome pathway (AOP) that can now be used for further studies and a starting point to build a more comprehensive AOP.

2. Dimethoate Adverse Outcome Pathway

As described previously, dimethoate is an organophosphate insecticide widely used, and its main toxic mechanism is the inhibition of the enzyme acetylcholinesterase, therefore causing alterations in the nervous system of organisms that will result in their death (Soreq and Seidman, 2001). The study performed showed not only neurotoxicity inherent to the negative effects of dimethoate upon non-target organisms, but also other mechanistic pathways and behavioural aspects not described previously.

2.1. Growth, moult and reproduction

Starting at a molecular level, dimethoate affects reproduction and growth genes like vitellogenin and chitinase, which are involved in growth, moult and reproduction. Along with these two genes it also affects haemocyanin, which is the protein responsible for the

transport of oxygen in isopods and that plays an important role also in moult processes (van Holde and Miller, 1995). Along with these changes at a molecular level is also possible to observe essential and non-essential amino acid changes at a metabolomic level, and changes in energy reserves (carbohydrates, lipids and proteins). All of these changes come in accordance with previous studies (e.g. Drobne and Štrus, 1996) that point out as stated previously changes in growth, moult and reproduction of this species of terrestrial isopods.

2.2. Oxidative Stress and detoxification

At a molecular level, dimethoate affected oxidative stress handling and detoxification genes like SOD, CYP450, GPx and GST. This impact is afterwards observed on the activity of enzymes encoded by some of the previous genes and also by the LPO rate, an indicator of oxidative stress damage. These results indicated a clear impact on the detoxification processes and cellular damage which *per se* will impact the energy budget of organisms. In a normal situation where organisms are not exposed to any stressor, their energy budget is used for their basal metabolism, reproduction, growth and responses to predators. If organisms are exposed to any stress (such as dimethoate) the energy budget will be also allocated for detoxification. This new requirement will lead to changes in the energy budget distribution throughout the other processes thus resulting in a higher vulnerability.

2.3. Energy metabolism

Along with the previous genes that indirectly affected their energy budgets, the LDH gene and its corresponded encoded enzyme activity, metabolites such as fumarate, ADP, ATP, energy consumption, energy available or CEA are also shown to be impaired.

2.4. Genetics, epigenetics and cell cycle impairment

The genes TOP2A, SIRT and TRRAP were also found to be affected by dimethoate. These genes are associated with transcription and epigenetic acetylation/deacetylation in histones and also in cell cycle mainly in DNA replication steps (Sawan et al., 2008; Wang, 2002). The impact observed in these genes can then be related to reproduction and also to an increase on mortality.

2.5. Neurotoxicity

The main target of the pesticide dimethoate is the inhibition of the enzyme acetylcholinesterase. In fact, this enzyme activity was inhibited by this pesticide along with an impact on its transcription observed by the gene that encodes the enzyme (Soreq and Seidman, 2001). Although no other genes related to other proteins involved in the same neurotransmission pathway were attained, it was possible to observe at a metabolomic level an impact in glutamine, glutamate and choline levels.

All the previous described mechanisms will lead overall to a decrease on the decline of the population by a decrease in reproduction and/or higher mortality, and also to a reduction of fitness in processes involving inter-species competition.

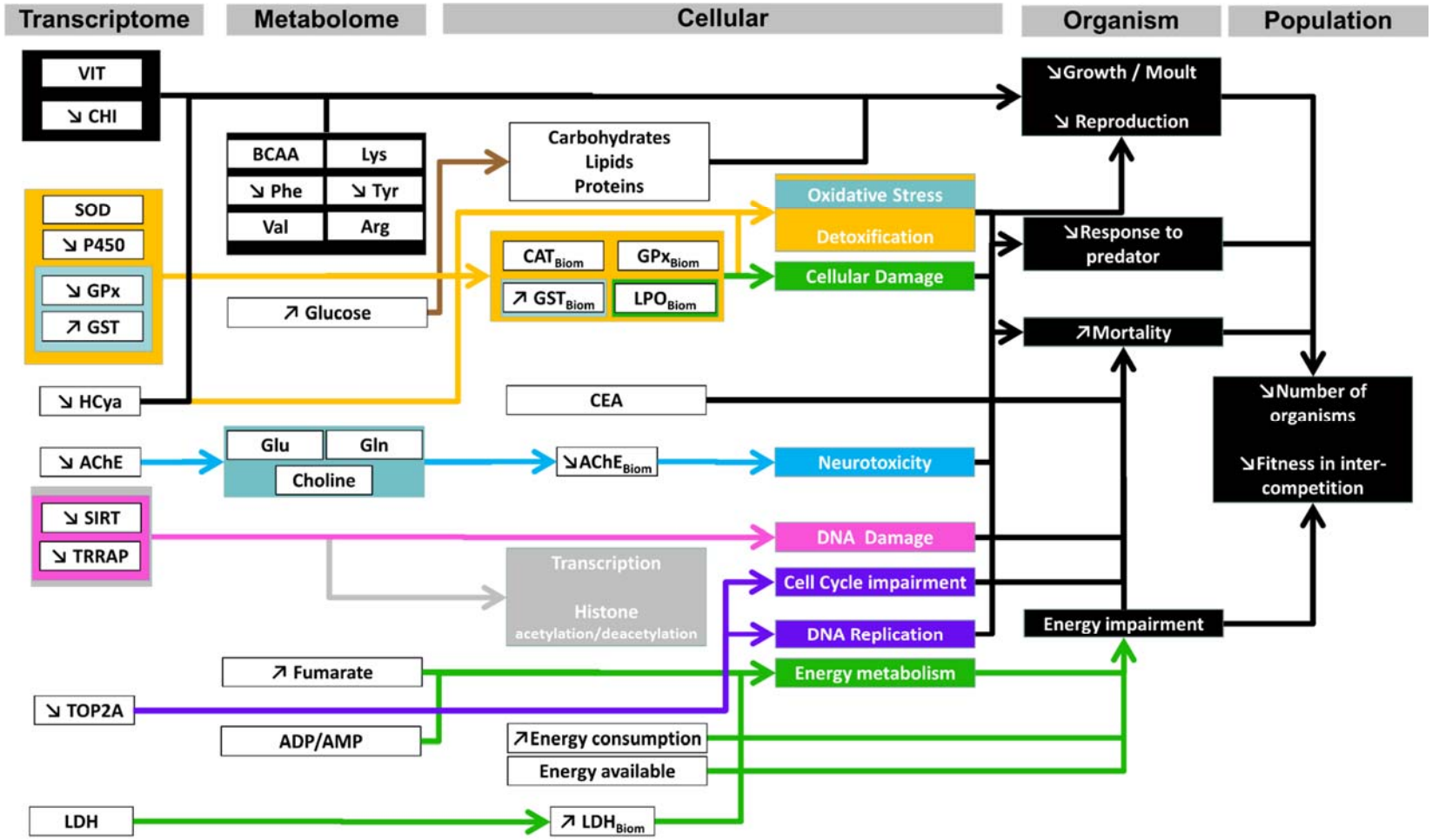

Fig. 8.1 Adverse outcome pathway draft for Porcellionides pruinosus, exposed to dimethoate. VIT- vitellogenin, CHI- chitinase 3, SOD- superoxide dismutase, P450- cytochrome P450, GPx- glutathione peroxidase; GPxBiom- glutathione peroxidase activity, GST- glutathione S-transferases, GSTBiom- glutathione S-transferases activity, Hcya- haemocyanin B chain, AChE- acetylcholinesterase, AChEBiom- acetylcholinesterase activity, SIRT- sirtuin 1, TRRAP- transformation/transcription domain-associated protein, TOP2A- topoisomerase (DNA) II alpha, LDH- L-lactate dehydrogenase, Glu- glutamate, BCAA- branched chain amino acids, Lys- lysine, Phe- phenylalanine, Tyr- tyrosine, Gln- glutamine, CAT- catalase activity, LPOBiom – lipid peroxidation rate, CEA- cellular energy allocation. White boxes correspond to measured parameters.

3. Nickel Adverse Outcome Pathway

Nickel is a micro-essential metal, which at high exposure levels can be considered carcinogenic (Vandenbrouck et al., 2011). The present study shows that nickel can cause directly or indirectly this carcinogenic effect and that its range of action varies from epigenetic and genetic factors to cell division, oxidative stress or reproduction impairment.

3.1. Epigenetic markers

Nickel showed to regulate a high number of genes that encode epigenetic markers such as hypermethylation, histone alteration or chromatin condensation. Some of those genes are: the mitotic spindle assembly checkpoint (MAD2A), a component of the spindle-assembly checkpoint, that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate; the DNA (cytosine-5-)-methyltransferase 1 (DNTMT1), responsible for the methylation of CpG residues; the SET domain containing lysine methyltransferase 7 (SETD7), an histone methyltransferase; and chromatin assembly factor 1, subunit B (CHAF1B), a complex that is thought to mediate chromatin assembly (Grabsch et al., 2003; Hermann et al., 2004; Sutherland et al., 2001). These epigenetic modifications, although considered flexible genomic parameters that can be developed over time, may prepare organisms to deal with a specific stressors, but also have an associated cost that can prejudice the organism and led to a decline on their population or a diminish in fitness if a fast change in environment is observed.

3.2. Oxidative stress and detoxification

As other metals, nickel induces the formation of ROS species and thus oxidative stress. The oxidative stress handling and detoxification genes like SOD, CYP450, GPx and GST along with the LPO rate (indicative of oxidative stress damage) appeared altered not only in their encoded genes, but also when evaluating their enzymatic activity. Also at a metabolomic

level, the alterations in glutamate and glycine levels are indicative of an impact in the formation of glutathione, which *per se* is essential for the activity of enzymes such as GST or GPx. As stated previously the impact of detoxification processes in the organisms' energy budget will lead to a higher vulnerability.

3.3. Energy metabolism

Identically to the AOP draft developed for dimethoate, the impact of nickel was also observed. The identified genes (LDH) and metabolites (ADP, AMP, fumarate) and the energy related parameters (Ea, Ec and CEA) play an identical role on the toxicity of these two model stressors and lead to the same energy budget impact leading, which can potentially culminate in a decline at the population level.

3.4. Growth, moult and reproduction

Genes like vitellogenin and chitinase, and those from the family of genes sodium-coupled monocarboxylate transporters (SLC), which belong to the solute carrier transports, appeared impaired. These genes are known to be involved in growth, moult and reproduction processes, and therefore are prone to induce also changes in population dynamics in exposure scenarios. The encoded gene for haemocyanin that plays an important role in moult also appears altered, as it was also observed for dimethoate exposures. At the metabolomic level the essential and non-essential amino acid changes and the changes in energy reserves (carbohydrates, lipids and proteins) also appear impacted thus previewing alterations in growth, moult and reproduction of isopods upon exposure.

3.5. Neurotoxicity

Some studies have reported metals affecting neurotransmission in organisms although a vast number of them is related to the inhibition of the enzyme AChE. In the case of nickel an inhibition of the gene that encodes AChE was observed, but also other genes related to the GABA receptors were also observed to be impaired. At the metabolic level, choline also appears impacted, thus supporting in the other findings related to neuro transmission processes. As consequence of these impairments responses to predators or higher mortalities can occur and thus resulting in an unbalanced population with also a lower fitness in inter-species competition.

3.6. Metal trafficking

Upon metal exposure, organisms will have specific proteins that are in charge of transporting and accumulate/excrete these elements according to their necessities. Although in the case of nickel no information is available regarding mechanistic processes of metal transportation and storage/elimination, in the specific case of terrestrial isopods, nickel appears to impact in zinc finger proteins and RING finger proteins (larger zinc finger proteins) by replacing the ion zinc. The increase in haemocyanin gene's transcription was also reported for other metals as a mechanism of transport (van Holde and Miller, 1995; Zatta, 1984) thus making possible that this gene may also be involved in nickel trafficking.

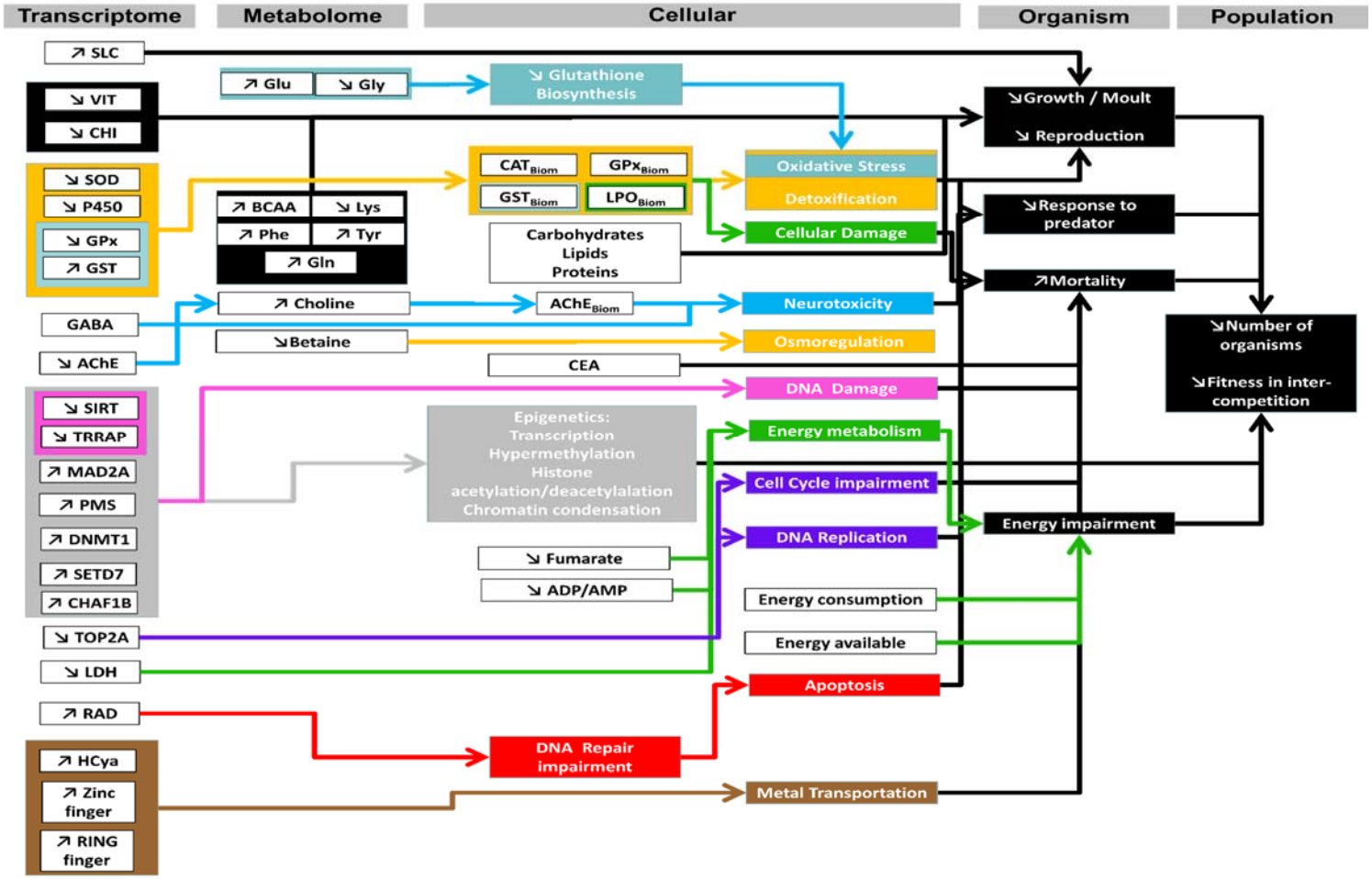

Fig. 8.2 Adverse outcome pathway draft for organisms from the species Porcellionides pruinosus, exposed to dimethoate. **VIT**- vitellogenin, **CHI**- chitinase 3, **SOD**- superoxide dismutase, **P450**- cytochrome P450, **GPx**- glutathione peroxidase; **GPxBiom**- glutathione peroxidase activity, **GST**- glutathione S-transferases, **GSTBiom**- glutathione S-transferases activity, **Hcya**- haemocyanin B chain, **AChE**- acetylcholinesterase, **AChEBiom**- acetylcholinesterase activity, **SIRT**- sirtuin 1, **TRRAP**- transformation/transcription domain-associated protein, **TOP2A**- topoisomerase (DNA) II alpha, **LDH**- L-lactate dehydrogenase, **Glu**- glutamate, **BCAA**- branched chain amino acids, **Lys**- lysine, **Phe**- phenylalanine, **Tyr**- tyrosine, **Gln**- glutamine, **CAT**- catalase activity, **LPOBiom** – lipid peroxidation rate, **CEA**- cellular energy allocation. **SLC**- sodium-coupled monocarboxylate transporters, **GABA**- gamma-aminobutyric acid receptors, **MAD2A**- mitotic spindle assembly checkpoint, **PMS**- mismatch repair endonuclease, **DNMT1**- DNA (cytosine-5-)-methyltransferase 1, **SETD7**- histone lysine N-methyltransferase 7, **CHAF1B**- chromatin assembly factor 1, subunit B, **RAD**- DNA repair and recombination proteins family. White boxes correspond to measured parameters.

In brief the present study used several multi organizational level endpoints to infer on the potential impact of dimethoate and nickel. The general adverse outcome pathways (AOP), although still in a very initial draft form, can be used as a base for their full development and application in further studies, as they *"can serve a number of ubiquitous purposes, including the establishment of (quantitative) structure-activity relationships, the development of novel in vitro toxicity screening tests and the elaboration of prioritization strategies."* as stated by Vinken (2013). The first approach of this study was based on the impact of several abiotic factors in terrestrial isopods and can be used as the foundation for further studies. Along with the AOP it is important to highlight that the present study presents an exposure followed by a recovery phase to allow the understanding of retrieval of effects. It also presents the first metabolomic profile of this isopod species and the annotation of metabolites (e.g. essential amino acids) whose acknowledgement was not available before, by using ^{1}H NMR techniques. In addition, the effects of a model pesticide and metal (that to our knowledge has not been previously reported for any other terrestrial isopod species) were also assessed in order to evaluate how the organism metabolism was reacting upon exposure. Finally this study also presents the first full body transcriptome of a terrestrial isopod, a transcriptome confining a great number of genes and that can be used further as a foundation for studies aiming at understanding chemical modes of action in these organisms. This approach was followed in a similar way, by the RNA-Seq analysis performed with organisms exposed to nickel and dimethoate, bringing new insights into the mechanisms of toxicity of these stressors which are still poorly known.

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SUPPLEMENTARY DATA

Supplementary Data – Chapter II

1. Materials and methods

1.1. Post-mitochondrial supernatant

The protocol to obtain the post-mitochondrial supernatant (PMS) that will be used for lipid peroxidation, glutathione *S*-transferases, glutathione peroxidase and catalase analysis was followed as described by Ferreira *et al.* (2010). Each replicate (two organisms) was homogenized using a sonicator (*Kika Labortechnik*, V200Scontrol, Germany) in 1ml K-Phosphate 0.1M buffer, pH 7.4. From the homogenate, 150µL were separated to a microtube and 5µL butylated hydroxytoluene (BHT) 4% in methanol were added for endogenous lipid peroxidation (LPO) determination. The remaining tissue homogenate (850 µL) was centrifuged at 10000g for 20 min. (4ºC) to isolate the Post-Mitochondrial Supernatant (PMS). The PMS was divided into four microtubes for posterior analysis of biomarkers and protein quantification. All microtubes were stored at -80ºC until analysis, for a period no longer than 2 weeks.

1.2. Lipid peroxidation

The lipid peroxidation (LPO) assay was based on the methods described by Bird and Draper (1984) and Ohkawa *et al.* (1979) and adapted to microplate by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 150 µL homogenated tissue, 500 µL trichloroacetic acid sodium salt (TCA) 12% (w/v), 500 µL 2-thiobarbituric acid (TBA) 0.73% (w/v) and 400µL Tris-HCl 60mM with diethylenetriaminepentaacetic acid (DTPA) 0.1mM. The reaction was carried out at 100ºC in a water bath for 1h. After this, samples were centrifuged for 5 min. at 11500 rpm (25ºC). Samples were kept away from light, at 25ºC and immediately read at 535 nm. LPO was expressed as nmol TBARS hydrolyzed per minute per mg of wet weight, using a molar extinction coefficient of 1.56 x10 M^{-1} cm^{-1}.

1.3. Glutathione S-Transferases

Glutathione *S*-Transferases (GST) activity was determined based on the method described by Habig *et al.* (1974). The PMS (100µL) was added to 200µL of a reaction solution and the result/substrate produced was measured at 340 nm. The reaction solution was a mixture of 4.95 ml K-phosphate buffer 0.1M (pH 6.5) with 900µL L-glutathione reduced (GSH) 10mM, and 150µL 1-chloro-2,4-dinitrobenzene (CDNB) 10mM. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to a nmol of substrate hydrolyzed / min, using a molar extinction coefficient of 9.6×10^{-3} M^{-1} cm^{-1}.

1.4. Glutathione Peroxidase

Glutathione Peroxidase (GPx) activity was determined based on the method described by Mohandas *et al.* (1984). PMS (50µl) was mixed with 840µl K-phosphate buffer 0.05 M (pH 7.0), in a EDTA 1 mM solution, sodium azide 1mM and glutathione redutase (GR) (7.5mL from stock with 1 U/mL). Then 50 µL glutathione reduced (GSH) 4mM, NADPH and H_2O_2 (10 µl, 0.5mM) was added as substrate to the solution. The decrease in NADPH content (50µl, 0.8mM) was measured at 340 nm and the enzymatic activity expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 6.22×10^{-3} M^{-1} cm^{-1}.

1.5. Catalase

Catalase (CAT) activity was determined based on the method described by Clairborne (1985). PMS (50µl) was mixed with 500µL H_2O_2 0.030M, and 950µL K-Phosphate 0.05M (pH 7.0) and the decomposition of the substrate (H_2O_2) measured at 240 nm. The enzymatic activity was expressed as unit (U) per mg of protein where a U corresponds to one µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

1.6. Acetylcholinesterase- sample preparation and reaction

One isopod head per sample was homogenized using a sonicator in 500µl of potassium phosphate buffer (0.1M, pH 7.2), and the supernatants obtained after centrifugation (4ºC, 3800g, 3 min) were removed and stored at -80ºC until enzymatic analysis. The AChE activity determination was performed according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996).

In a 96 well microplate 250 µl of the reaction solution was added to 50µl of the sample and the absorbance was read at 414 nm, after 10, 15 and 20min. The reaction solution had 1ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10mM solution, 1.280ml of 0.075M acetylthiocholine iodide solution and 28.920 ml of 0.1M phosphate buffer. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $1.36x10^{-3}$ M^{-1} cm^{-1}.

1.7. Protein quantification for biomarkers

For all biomarker, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

1.8. Energy reserves:

To determine total protein, carbohydrate and lipid contents, one isopod was homogenized using a sonicator in 1000µl distilled water after which was divided into three microtubes each one containing 300µl of the homogenate. The first fraction was used to determine protein and carbohydrate content, the second fraction to determine lipid content and the third fraction to determine the electron transport activity (ETS).

To determine total protein and carbohydrate contents, the homogenate was mixed with 100µl of 15% trichloroacetic acid (TCA) and incubated at -20ºC for 10 min (adapted from de Coen and Janssen (1997). After centrifugation (1000g, 10 min, 4ºC), the supernatant was separated as well as the carbohydrate fraction. The remaining pellet was resuspended in 1250µl sodium hydroxide (NaOH), incubated at 60 ºC for 30 min, after which it was neutralised with 750µl hydrocloric acid (HCl) and used as the protein fraction. Total protein content was then determined using the Bradford's reagent (Bradford 1976), and by measuring the absorbance at 590 nm using bovine serum albumin as a standard. Total carbohydrate content was determined by adding 50µl of 5% phenol and 200µl sulphuric acid (H_2SO_4) to 50µl of sample in a multiwell microplate, incubated for 30min at 20 ºC; the absorbance was measured at 492 nm using glucose as a standard. The protein and carbohydrate content is expressed as mg/mg organism (org) and J/mg org (expressed as fresh weight).

Total lipid quantification was based in the method described by Bligh and Dyer (1959). To the homogenate obtained was added 500µl chloroform (spectrofotometric grade). After vortexed, 500µl methanol (spectrofotometric grade) and 250µl bi-distilled water were added, centrifuged (1000g, 5min, 4ºC) and the top phase removed; the remaining phase was used for lipid measurement. 100µl of lipid extract plus 500µl H_2SO_4 were heated for 15 min (200ºC); after cooling down, 1.5ml of bi-distilled water was added and the total lipid content determined by measuring the absorbance at 375 nm using tripalmitin as a standard. The lipid content was expressed as mg/ mg org and J/mg org (expressed as fresh weight).

The electron transport activity was measured following de Coen and Janssen (1997) with the following modifications. To the homogenate obtained was added 150 µL of a buffer (0.3 M Tris-HCl pH 8.5, 45% (w/v) Poly Vinyl Pyrrolidone, 459 µM $MgSO_4$ and 0.6% (w/v) Triton X-100). Samples were then centrifuged at 1300 g during 10 min (4ºC).

The electron transport activity was determined by adding 50 µL of sample to 150 µL buffered substrate solution (0.13M Tris HCl, 0.3% (w/v) Triton X-100, pH 8.5, 1.7 mM NADH and 250 µM NADPH). The reaction was started by adding 100 µL INT (p-IodoNitroTetrazolium; 8 mM) and the absorbance measured at 490nm for 3 min.

The amount of formazan formed was calculated using a molar extinction coefficient of $15900 M^{-1} cm^{-1}$.

1.9. Cellular Energy Allocation (CEA)

The different energy reserve fractions (Ea): protein, carbohydrate and lipids obtained for the individual organisms were transformed into energetic equivalents using the energy of combustion described by Gnaiger (1983): 17.5 J/mg carbohydrate, 24 J/mg protein and 39.5 J/mg lipid. The cellular respiration rate (Ec) was determined using the ETS data, based on the theoretical stoichiometric relationship that for each 2 μmol of formazan formed, 1 μmol of O2 was consumed in the ETS system. The oxygen consumed per isopod was transformed into energetic equivalents using the specific oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture of 484 kJ/mol O2 (Gnaiger, 1983). The Ea, Ec and CEA value were calculated as described by Verslycke *et al.* (2004):

Ea (available energy) = carbohydrates + lipids + proteins (mJ / mg org.)
Ec (energy consumption) = ETS activity (mJ / mg org. / h)
CEA (cellular energy allocation) = Ea/Ec (/h)

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Supplementary Data – Chapter III

1. Materials and methods

1.1. Post-mitochondrial supernatant

The protocol to obtain the post-mitochondrial supernatant (PMS) that will be used for lipid peroxidation, glutathione *S*-transferases, glutathione peroxidase and catalase analysis was followed as described by Ferreira *et al.* (2010). Each replicate (two organisms) was homogenized using a sonicator (*Kika Labortechnik*, V200Scontrol, Germany) in 1ml K-Phosphate 0.1M buffer, pH 7.4. From the homogenate, 150µL were separated to a microtube and 5µL butylated hydroxytoluene (BHT) 4% in methanol were added for endogenous lipid peroxidation (LPO) determination. The remaining tissue homogenate (850 µL) was centrifuged at 10000g for 20 min. (4ºC) to isolate the Post-Mitochondrial Supernatant (PMS). The PMS was divided into four microtubes for posterior analysis of biomarkers and protein quantification. All microtubes were stored at -80ºC until analysis, for a period no longer than 2 weeks.

1.2. Lipid peroxidation

The lipid peroxidation (LPO) assay was based on the methods described by Bird and Draper (1984) and Ohkawa *et al.* (1979) and adapted to microplate by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 150 µL homogenated tissue, 500 µL trichloroacetic acid sodium salt (TCA) 12% (w/v), 500 µL 2-thiobarbituric acid (TBA) 0.73% (w/v) and 400µL Tris-HCl 60mM with diethylenetriaminepentaacetic acid (DTPA) 0.1mM. The reaction was carried out at 100ºC in a water bath for 1h. After this, samples were centrifuged for 5 min. at 11500 rpm (25ºC). Samples were kept away from light, at 25ºC and immediately read at 535 nm. LPO was

expressed as nmol TBARS hydrolyzed per minute per mg of wet weight, using a molar extinction coefficient of 1.56 x10 M^{-1} cm^{-1}.

1.3. Glutathione S-Transferases

Glutathione *S*-Transferases (GST) activity was determined based on the method described by Habig *et al.* (1974). The PMS (100µL) was added to 200µL of a reaction solution and the result/substrate produced was measured at 340 nm. The reaction solution was a mixture of 4.95 ml K-phosphate buffer 0.1M (pH 6.5) with 900µL L-glutathione reduced (GSH) 10mM, and 150µL 1-chloro-2,4-dinitrobenzene (CDNB) 10mM. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6 $x10^{-3}$ M^{-1} cm^{-1}.

1.4. Glutathione Peroxidase

Glutathione Peroxidase (GPx) activity was determined based on the method described by Mohandas *et al.* (1984). PMS (50µl) was mixed with 840µl K-phosphate buffer 0.05 M (pH 7.0), in a EDTA 1 mM solution, sodium azide 1mM and glutathione redutase (GR) (7.5mL from stock with 1 U/mL). Then 50 µL glutathione reduced (GSH) 4mM, NADPH and H_2O_2 (10 µl, 0.5mM) was added as substrate to the solution. The decrease in NADPH content (50µl, 0.8mM) was measured at 340 nm and the enzymatic activity expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 6.22$x10^{-3}$ M^{-1} cm^{-1}.

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Catalase (CAT) activity was determined based on the method described by Clairborne (1985). PMS (50µl) was mixed with 500µL H_2O_2 0.030M, and 950µL K-Phosphate 0.05M (pH 7.0) and the decomposition of the substrate (H_2O_2) measured at 240 nm. The enzymatic

activity was expressed as unit (U) per mg of protein where a U corresponds to one μmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

1.6. Lactate dehydrogenase- sample preparation and reaction

Lactate dehydrogenase (LDH) activity was determined at 340 nm by the method of Vassault (1983) adapted to microplate by Diamantino *et al.* (2001). One organism (excluding the head) was homogenized using a sonicator in 500 μl of Tris/NaCl buffer (0.1M, pH 7.2), and the supernatants obtained after centrifugation (4 ºC, 4200g, 3 min) were removed and stored at -80ºC until enzymatic analysis. Activity determinations were made using 40μL of sample and 250μL of NADH (0.24mM) and 40μL of piruvate (10mM). The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to one μmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $6.3x10^{-3}$ M^{-1} cm^{-1}.

1.7. Acetylcholinesterase- sample preparation and reaction

One isopod head per sample was homogenized using a sonicator in 500μl of potassium phosphate buffer (0.1M, pH 7.2), and the supernatants obtained after centrifugation (4ºC, 3800g, 3 min) were removed and stored at -80ºC until enzymatic analysis. The AChE activity determination was performed according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996).

In a 96 well microplate 250 μl of the reaction solution was added to 50μl of the sample and the absorbance was read at 414 nm, after 10, 15 and 20min. The reaction solution had 1ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10mM solution, 1.280ml of 0.075M acetylthiocholine iodide solution and 28.920 ml of 0.1M phosphate buffer. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $1.36x10^{-3}$ M^{-1} cm^{-1}.

1.8. Protein quantification for biomarkers

For all biomarker, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

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To determine total protein, carbohydrate and lipid contents, one isopod was homogenized using a sonicator in 1000µl distilled water after which was divided into three microtubes each one containing 300µl of the homogenate. The first fraction was used to determine protein and carbohydrate content, the second fraction to determine lipid content and the third fraction to determine the electron transport activity (ETS).

To determine total protein and carbohydrate contents, the homogenate was mixed with 100µl of 15% trichloroacetic acid (TCA) and incubated at -20ºC for 10 min (adapted from de Coen and Janssen (1997). After centrifugation (1000g, 10 min, 4ºC), the supernatant was separated as well as the carbohydrate fraction. The remaining pellet was resuspended in 1250µl sodium hydroxide (NaOH), incubated at 60 ºC for 30 min, after which it was neutralised with 750µl hydrocloric acid (HCl) and used as the protein fraction. Total protein content was then determined using the Bradford's reagent (Bradford 1976), and by measuring the absorbance at 590 nm using bovine serum albumin as a standard. Total carbohydrate content was determined by adding 50µl of 5% phenol and 200µl sulphuric acid (H_2SO_4) to 50µl of sample in a multiwell microplate, incubated for 30min at 20 ºC; the absorbance was measured at 492 nm using glucose as a standard. The protein and carbohydrate content is expressed as mg/mg organism (org) and J/mg org (expressed as fresh weight).

Total lipid quantification was based in the method described by Bligh and Dyer (1959). To the homogenate obtained was added 500µl chloroform (spectrofotometric grade). After

vortexed, 500µl methanol (spectrofotometric grade) and 250µl bi-distilled water were added, centrifuged (1000g, 5min, 4ºC) and the top phase removed; the remaining phase was used for lipid measurement. 100µl of lipid extract plus 500µl H_2SO_4 were heated for 15 min (200ºC); after cooling down, 1.5ml of bi-distilled water was added and the total lipid content determined by measuring the absorbance at 375 nm using tripalmitin as a standard. The lipid content was expressed as mg/ mg org and J/mg org (expressed as fresh weight).

The electron transport activity was measured following de Coen and Janssen (1997) with the following modifications. To the homogenate obtained was added 150 µL of a buffer (0.3 M Tris-HCl pH 8.5, 45% (w/v) Poly Vinyl Pyrrolidone, 459 µM $MgSO_4$ and 0.6% (w/v) Triton X-100). Samples were then centrifuged at 1300 g during 10 min (4ºC).

The electron transport activity was determined by adding 50 µL of sample to 150 µL buffered substrate solution (0.13M Tris HCl, 0.3% (w/v) Triton X-100, pH 8.5, 1.7 mM NADH and 250 µM NADPH). The reaction was started by adding 100 µL INT (p-IodoNitroTetrazolium; 8 mM) and the absorbance measured at 490nm for 3 min.

The amount of formazan formed was calculated using a molar extinction coefficient of $15900 M^{-1} cm^{-1}$.

1.10. Cellular Energy Allocation (CEA)

The different energy reserve fractions (Ea): protein, carbohydrate and lipids obtained for the individual organisms were transformed into energetic equivalents using the energy of combustion described by Gnaiger (1983): 17.5 J/mg carbohydrate, 24 J/mg protein and 39.5 J/mg lipid. The cellular respiration rate (Ec) was determined using the ETS data, based on the theoretical stoichiometric relationship that for each 2 µmol of formazan formed, 1 µmol of O2 was consumed in the ETS system. The oxygen consumed per isopod was transformed into energetic equivalents using the specific oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture of 484 kJ/mol O2 (Gnaiger, 1983). The Ea, Ec and CEA value were calculated as described by Verslycke *et al.* (2004):

Ea (available energy) = carbohydrates + lipids + proteins (mJ / mg org.)
Ec (energy consumption) = ETS activity (mJ / mg org. / h)
CEA (cellular energy allocation) = Ea/Ec

1.11. Integrated Biomarker Response (IBR)

To try to integrate all results from different biomarkers and understand global/general responses, the integrated biomarker response (IBR) was calculated according to Beliaeff and Burgeot (2002). The IBR is calculated by summing up triangular Star Plot areas calculated for each two neighbouring data (biomarkers and energy reserves, time or temperature).

To calculate the IBR for biomarkers and energy reserves, the general mean (m) and the standard deviation (s) of all data regarding a given biomarker was calculated, followed by a standardization for each situation to obtain Y, where $Y = (X - m)/s$, and X is the mean value for the biomarker at a given concentration. Then Z was calculated using $Z = -Y$ or $Z = Y$, in the case of a biological effect corresponds respectively to an inhibition or a stimulation. Regarding the biological effect considered for each parameter, AChE and the energy related parameters: lipids, carbohydrates, proteins, energy available (Ea) and CEA were all assumed to decrease upon dimethoate exposure. In a similar way LPO rate was always assumed to increase with the exposure to the pesticide. The energy consumption (Ec) and LDH can either increase or decrease depending on the intensity of the stressor, and with organisms' strategy as well. In theory, organisms tend to spend more energy in order to deal with stressor, but an opposite strategy can also be used. In this case, organisms tend to decrease the energy that is directed to other physiological processes and thus resulting in an overall lower consumption, even lower than in situations where they were not under stress. In a similar way, the activity of the biomarkers GST and CAT can also be induced in order to cope with the formation of lipid peroxides or inactivated by ROS-mediated denaturation (Lizawa et al. 1994). For these reasons, their kinetics must be followed through time to consider their biological effect.

The score (S) was calculated by $S = Z+|Min|$, where $S \geq 0$ and $|Min|$ is the absolute value for the minimum value for all calculated Y in a given biomarker at all measurements made.

Star plots were then used to display Score results (S) and to calculate the integrated biomarker response (IBR) as:

$$IBR = \sum_{i=1}^{n} A_i$$

$$A_i = \frac{S_i}{2} \sin\beta \, (S_i \cos\beta + S_{i+1} \sin\beta)$$

$$\beta = \tan^{-1}\left(\frac{S_{i+1} \sin\alpha}{S_i - S_{i+1} \cos\alpha}\right)$$

where S_i and S_{i+1} are two consecutive clockwise scores (radius coordinates) of a given star plot; A_i corresponds to the area the connecting two scores; n the number of biomarkers and energy reserves used for calculations; and $\alpha = 2\pi/n$.

In some sampling times, due to the high mortality obtained, it was not possible to determine all the parameters. Since the IBR is obtained by summing up all the parameters, to allow a correct and more accurate comparison it was divided by the number of sampling times and presented as IBR/n (Broeg and Lehtonen, 2006). Using this method it is possible to get an overall state of organisms for each parameter and each sampling time. As an example, the control for the exposure to 25ºC and the biomarker AChE, 10 time samples were obtained (0h, 24h, 48h, 96h, 7-days, 14-days, 21-days, 28-days, 35-days and 42-days) but for the highest concentration (10 mg dimethoate / kg soil) only 6 time samplings were possible to achieve data from (0h, 24h, 48h, 96h, 7-days and 14-days). So for the control, the IBR was divided by 10 giving IBR/n = 7.53/10 = 0.75 and for the highest exposure (10 mg dimethoate /kg) the IBR was divided by 6 giving IBR/n = 21.32/6 = 3.55.

Analysing the scores as a fitness index, values that differed in 0.5 from the control score were considered to be from an organism with a higher or lower fitness (higher or lower scores, respectively).

The IBR calculations were always performed with the same order of parameters for all sampling times: the neurotoxicity biomarker AChE (the enzyme for which the pesticide was designed to inhibit), followed by the detoxification and oxidative stress biomarkers GST, LPO and CAT, then the energy related biomarker LDH to serve as transition between biomarkers and energy related parameters and finally the lipids, carbohydrates and proteins content, the energy available (Ea), the energy consumption (Ec) and the CEA that integrates the last two parameters.

2. Results

2.1. Exposure to 20ºC

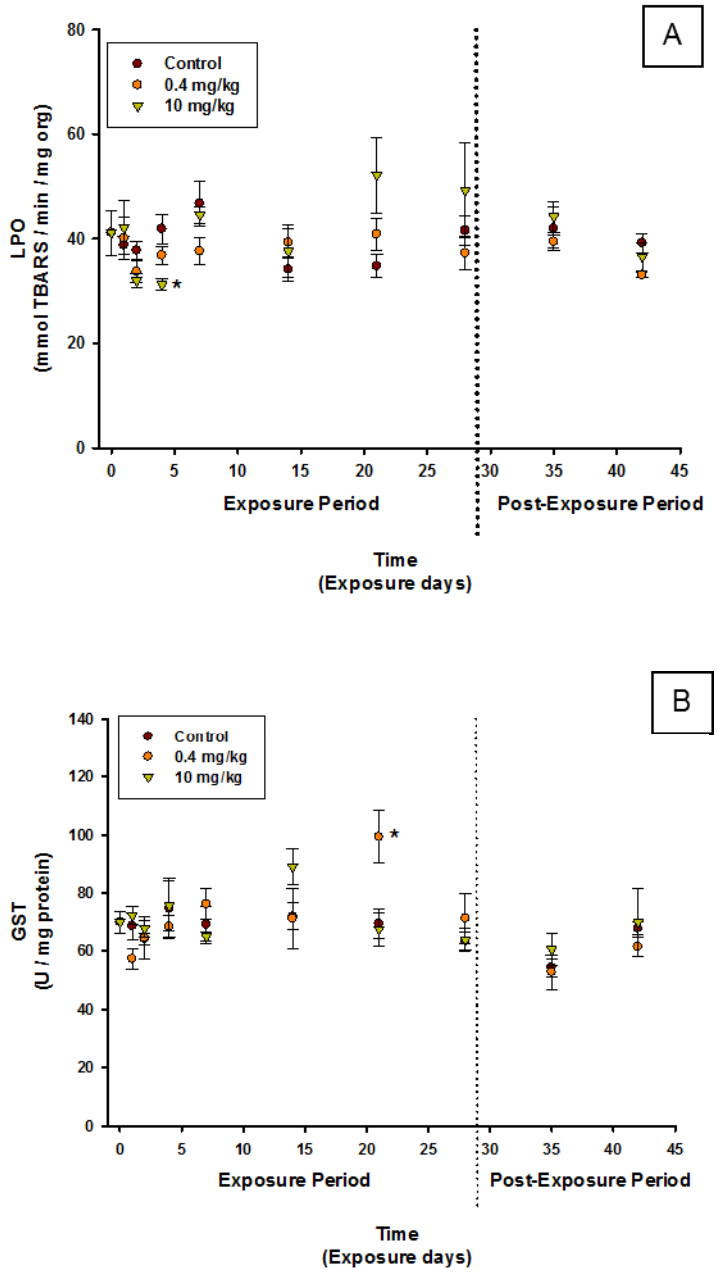
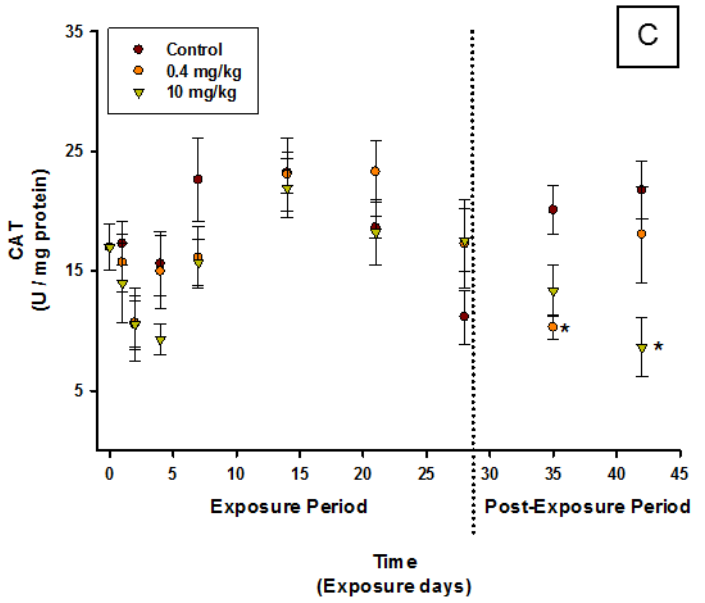


Fig. 1SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 20ºC for a 28 days period followed by a 14 days

recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$



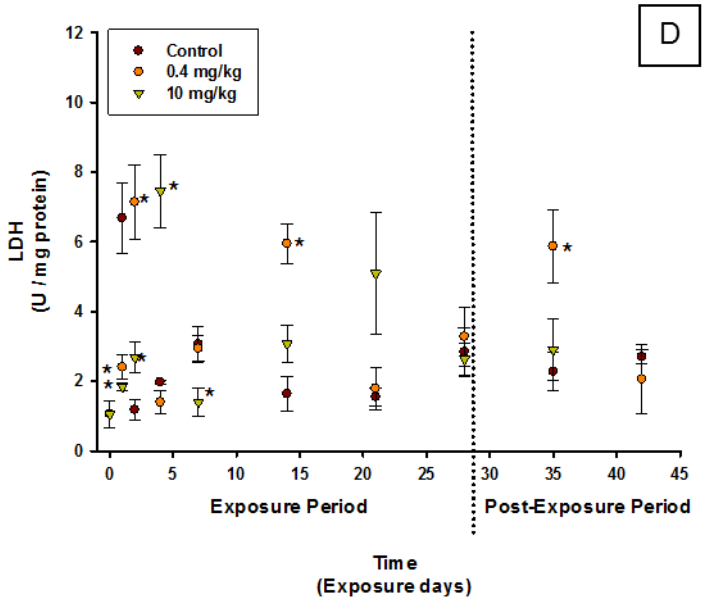


Fig. 1SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 20ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$

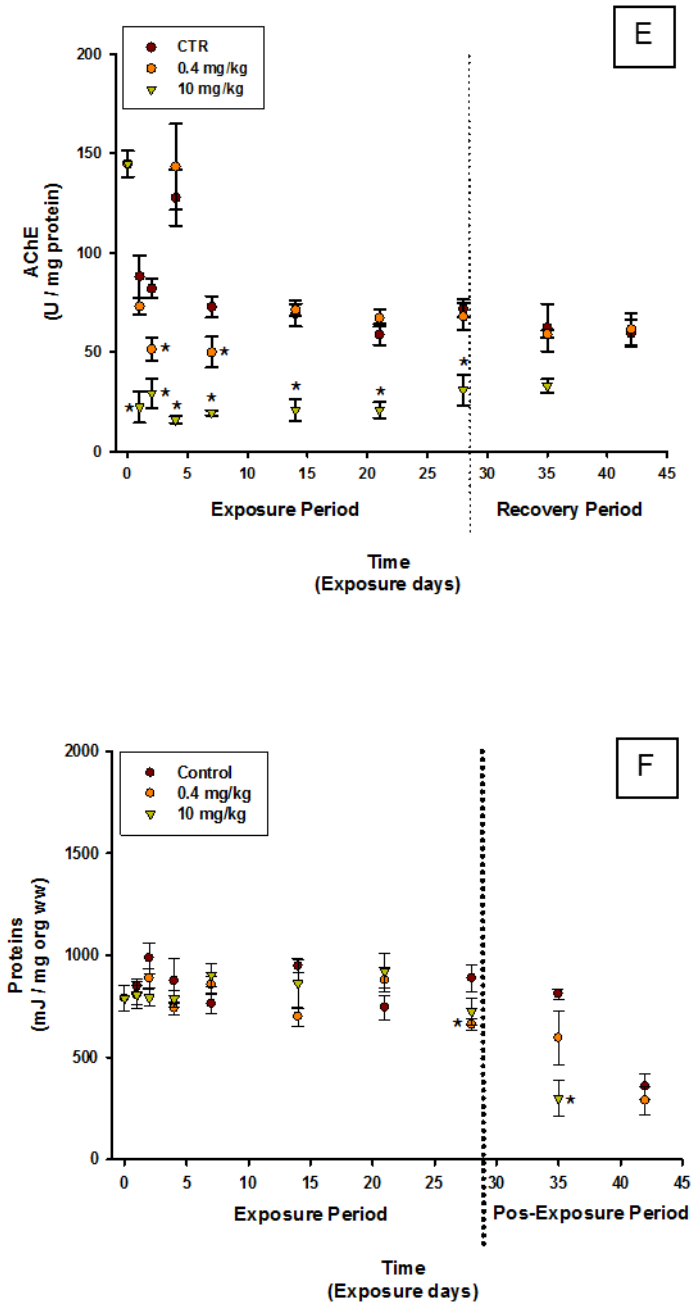


Fig. 1SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 20ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$

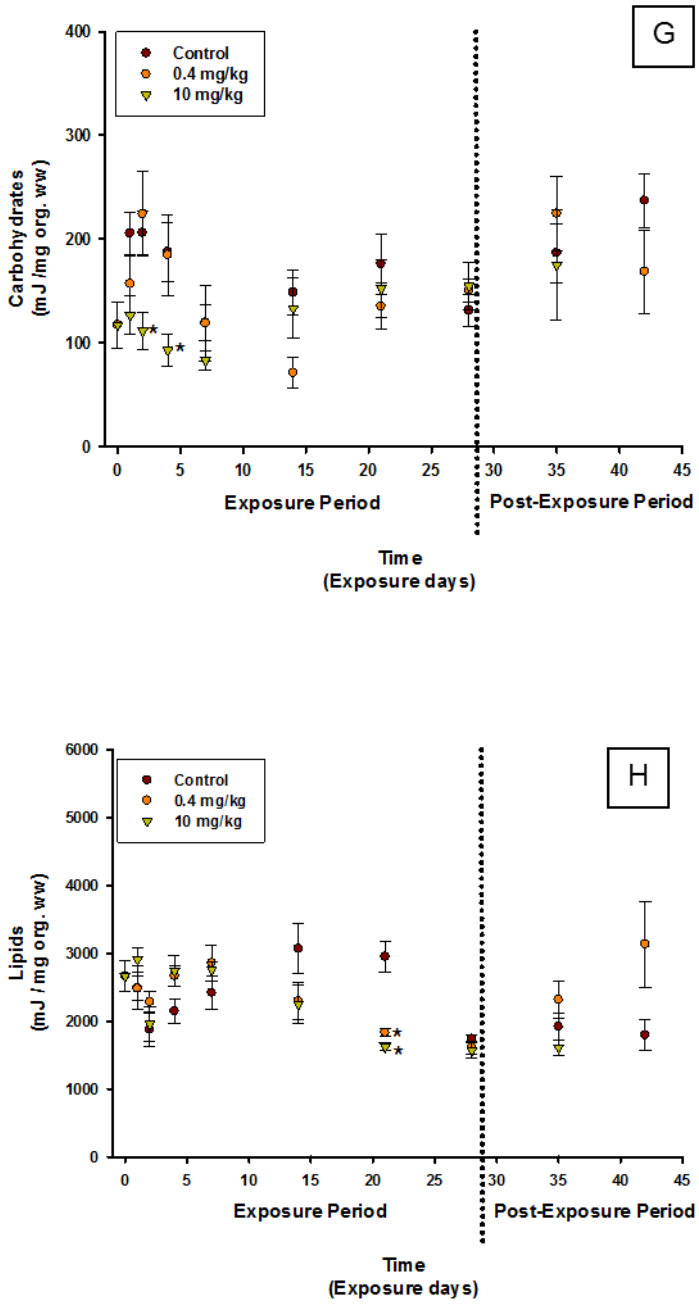
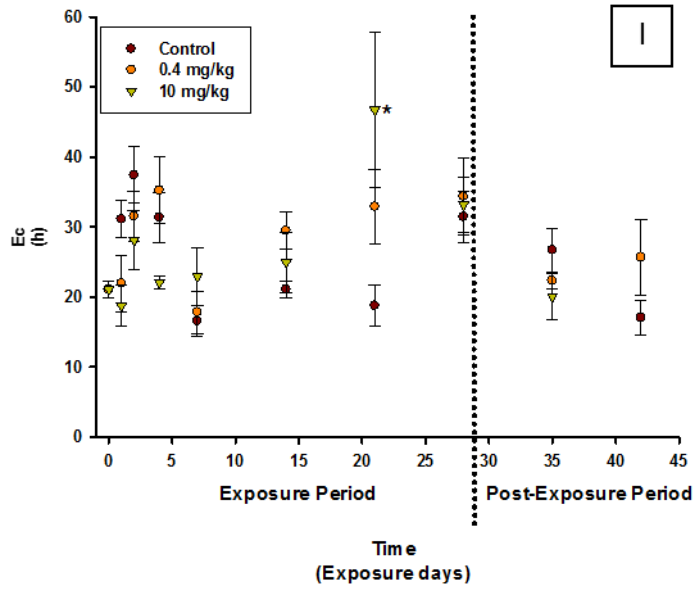


Fig. 1SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 20ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$



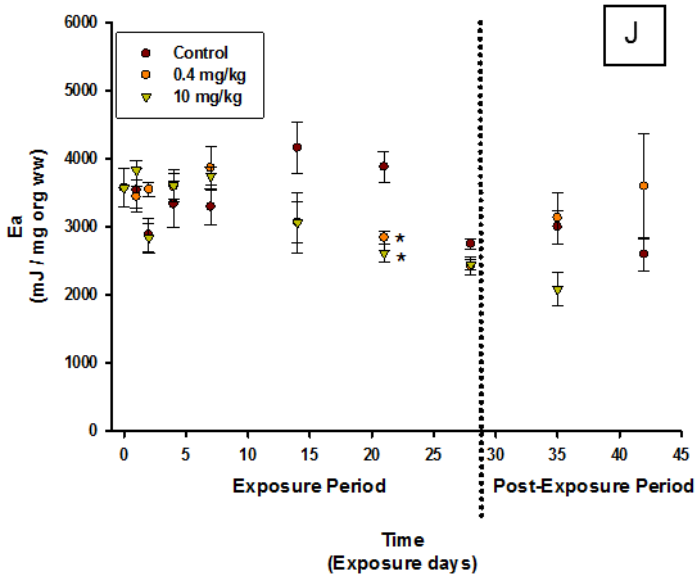
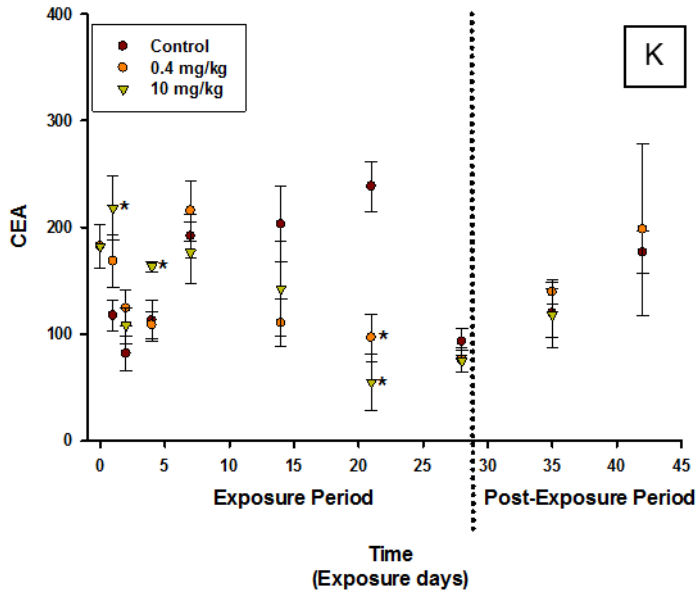


Fig. 1SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 20ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$



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2.2. Exposure to 25ºC

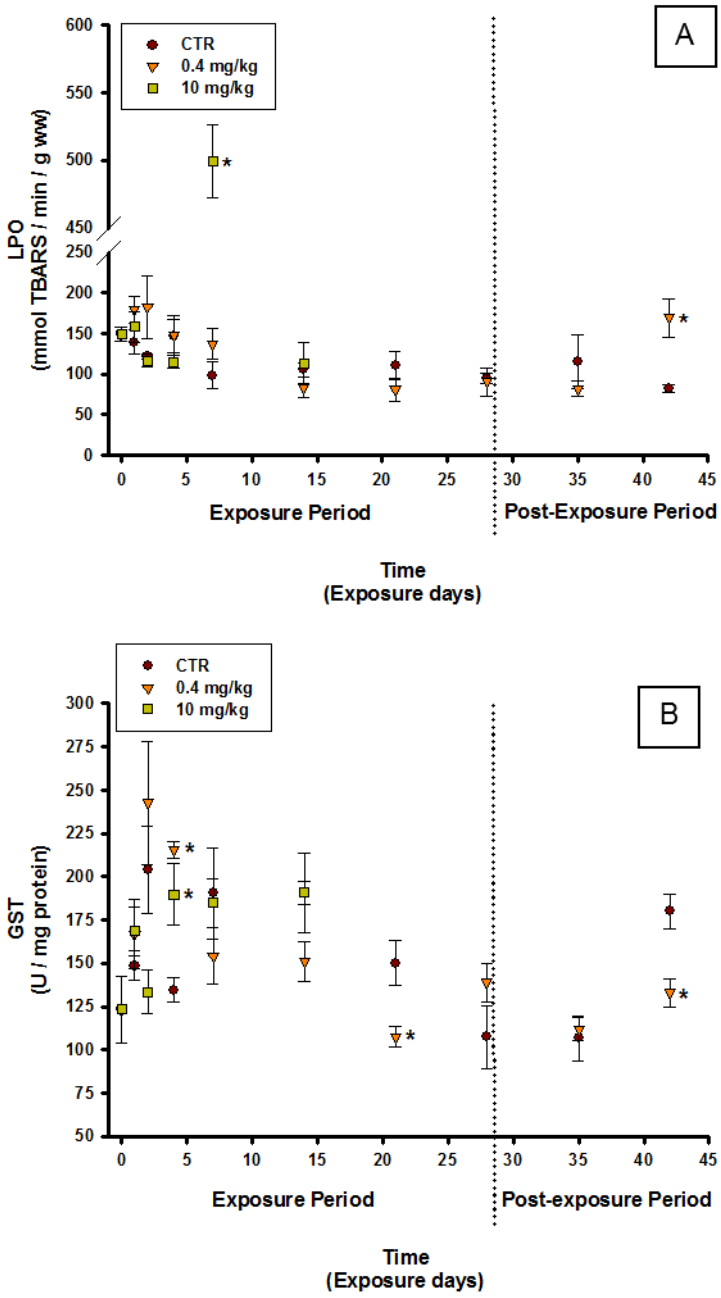

Fig. 2SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 25ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$

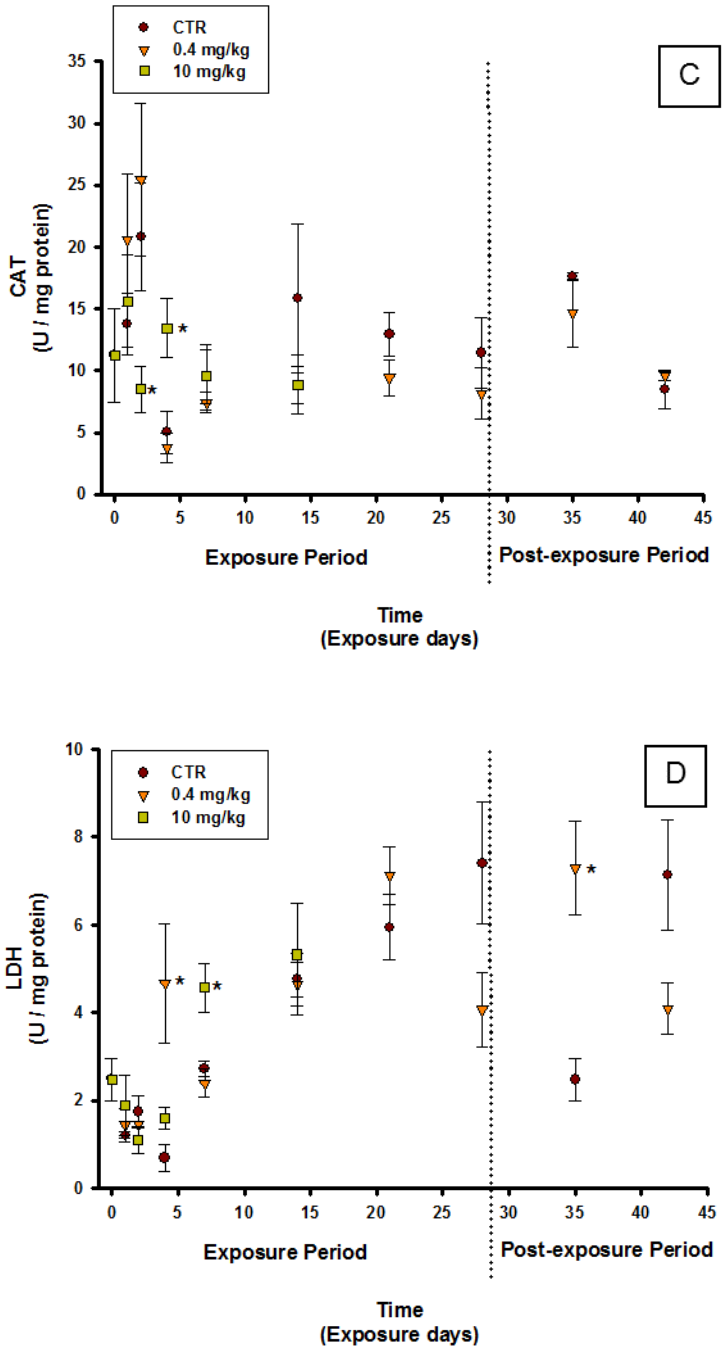

Fig. 2SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 25ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$

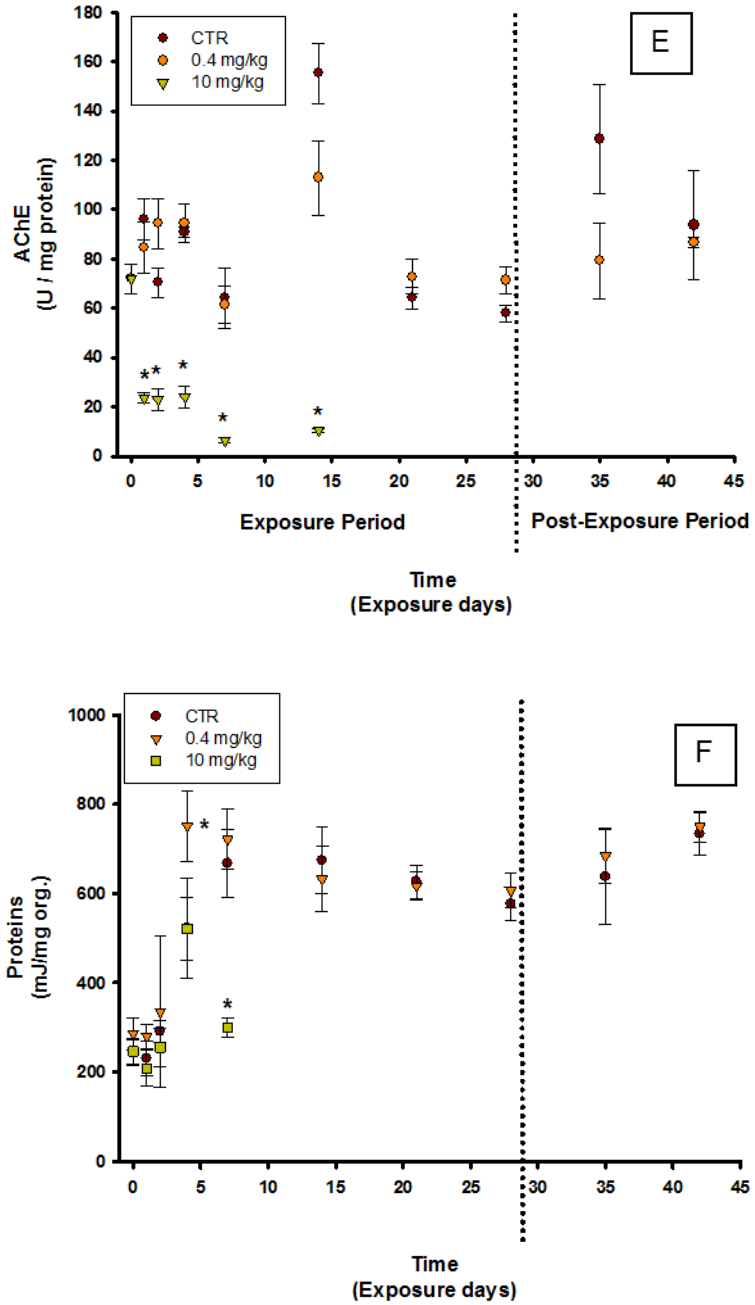

Fig. 2SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 25ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$

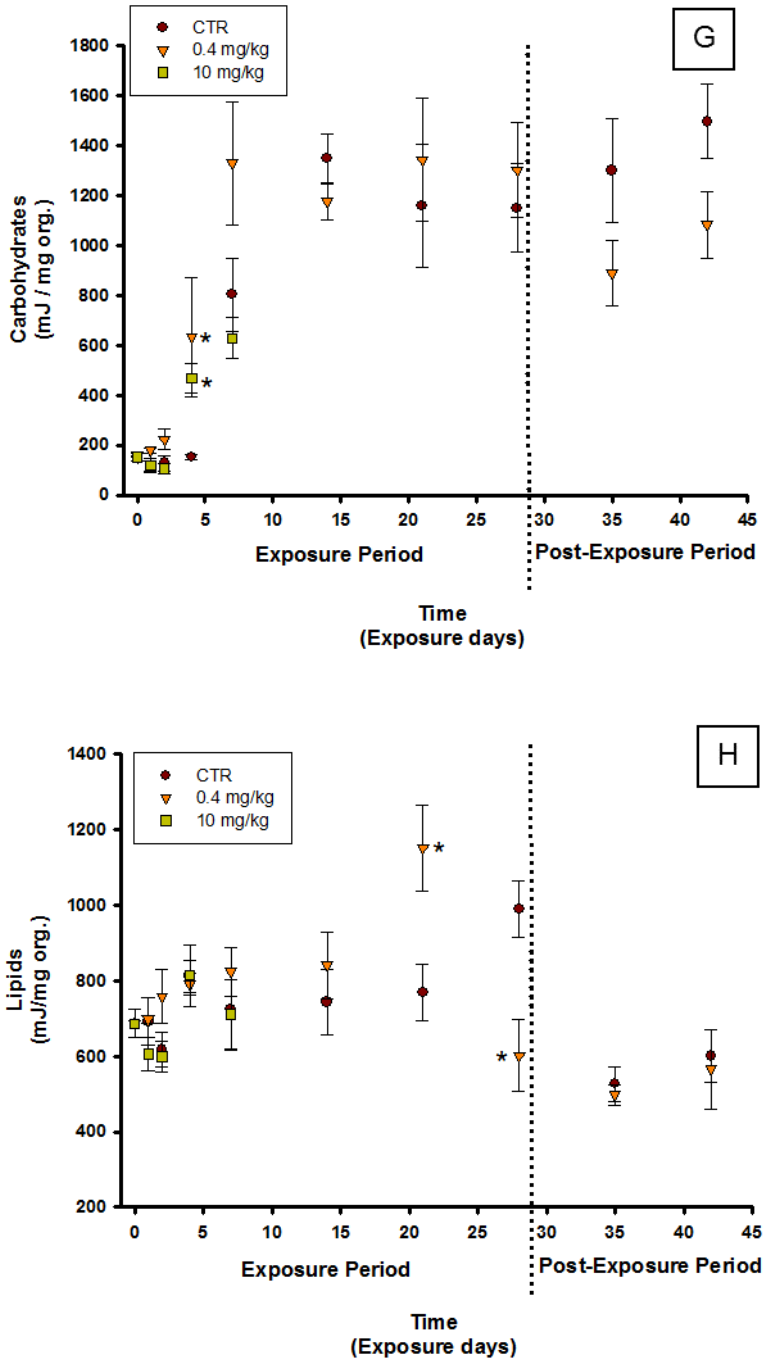

Fig. 2SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 25ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$

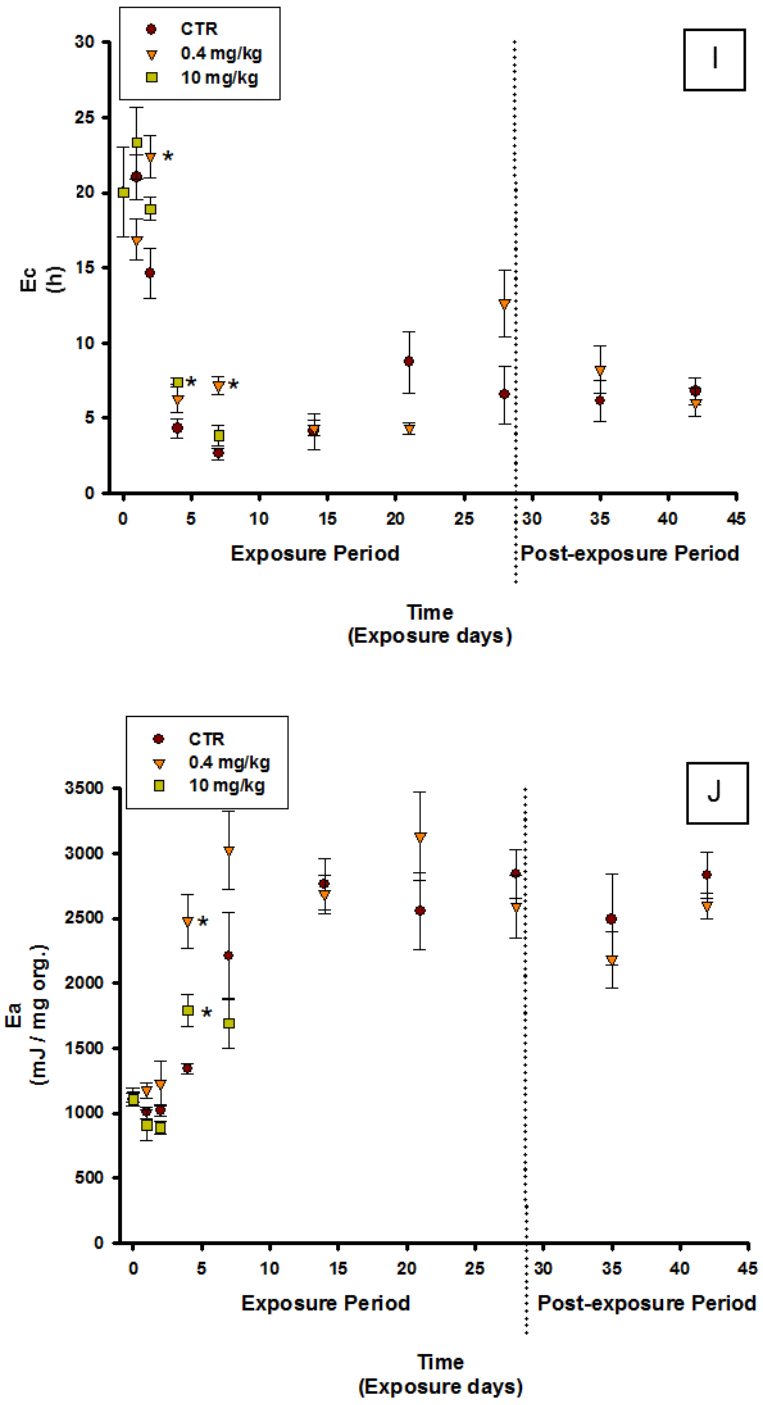

Fig. 2SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 25ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$

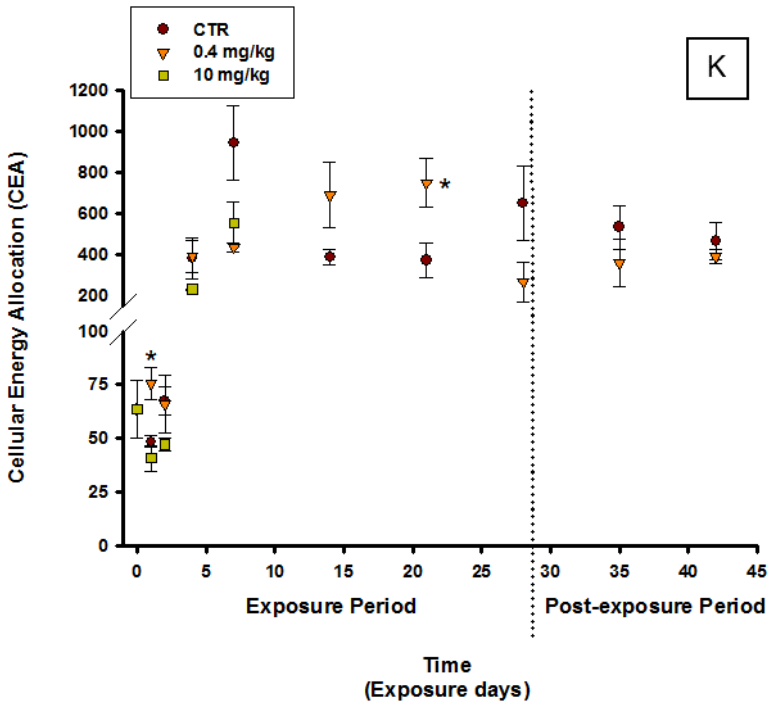

Fig. 2SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed dimethoate (Δ:0.4 mg / kg soil and □:10 mg / kg soil) at 25ºC for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$

2.3. Tables

Table 1SD IBR data comparison for biomarkers and energy reserves between control organisms from the species Porcellionides *pruinosus* and those exposed to dimethoate at 20ºC and 25ºC. Data refers to a 28 day exposure period followed by a 14 day recovery period. Red boxes denote deleterious effects, green boxes denote positive effects. □ - denotes increase, □ - denotes decrease; IBR is ± 0.5 from control's IBR value.

			AChE		GST		LPO		CAT		LDH		Lipids		Carbohydr.		Proteins		Ea		Ec		CEA	
			20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC
Exposure	24h	0.4 mg/kg			↘					↘	↘				↘			↗			↘	↗	↗	
		10mg/kg	↘	↘		↘			↘		↘		↗	↘	↘				↗		↘		↗	
	48h	0.4 mg/kg	↘	↗		↘	↗	↘		↗	↘		↗	↗			↘		↗		↘	↘	↗	
		10mg/kg	↘	↘		↗	↗			↘	↘				↘		↘				↘	↘	↗	
	96h	0.4 mg/kg				↘	↗			↘		↘	↗			↗	↘	↗		↗	↘			
		10mg/kg	↘	↘	↘	↘	↗		↘	↘	↘		↗		↘	↗		↗	↗	↗	↘		↗	
	7d	0.4 mg/kg	↘			↘	↗		↘				↗	↗		↗	↗	↗	↗			↘		↘
		10mg/kg	↘	↘	↘			↘	↘		↘	↘	↗		↘		↗	↘	↗	↘	↘			↘
	14d	0.4 mg/kg		↘		↘	↘			↘	↘		↘	↗	↘		↘		↘		↘		↘	↘
		10mg/kg	↘	↘	↘		↘			↘	↘		↘						↘		↘		↘	
	21d	0.4 mg/kg			↘	↘	↘		↘	↘		↘	↘	↗	↘		↗		↘	↗	↘	↘	↘	↗
		10mg/kg	↘				↘				↘		↘		↘		↗		↘		↘		↘	
	28d	0.4 mg/kg			↘	↘	↗		↘	↘		↘		↘			↘		↘			↘		↘
		10mg/kg	↘				↘		↘						↗		↘		↘					
Post-Exposure	7d	0.4 mg/kg		↘			↗		↘	↘	↗	↘	↗		↗	↘	↘				↘			↘
		10mg/kg	↘		↘								↘				↘		↘		↘			
	14d	0.4 mg/kg			↘	↘	↗	↘				↘	↗		↘	↘			↗		↘			
		10mg/kg																						

Table 2SD Statistical details for biomarkers and energy reserves of *Porcellionides pruinosus* between control and dimethoate exposed organisms at 20ºC and 25ºC for a 28 day exposure period followed by a 14 day recovery period. Data refers to *F* and respective *p* values from One Way ANOVA, *H* and *p* values from Kruskal Wallis ANOVA, *t* and *p* values from Student's t-tests.

			AChE		GST		LPO		CAT		LDH	
			20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC
Exposure	24h	0.4 mg/kg	F=18.411; p<0.001	F=67.438; p<0.001							F=19.509; p<0.001	
		10mg/kg										
	48h	0.4 mg/kg	F=17.738; p<0.001	F=24.468; p<0.001						F=5.895; p=0.023	F=15.896: p<0.001	
		10mg/kg										
	96h	0.4 mg/kg	F=26.097; p<0.001	F=49.940; p<0.001		F=9.815; p=0.004	F=7.471; p=0.008			F=7.027 ; p=0.015	F=17.401; p<0.001	F=10.722; p=0.004
		10mg/kg										
	7d	0.4 mg/kg	F=30.502; p<0.001	F=14.767; p<0.001				F=105.448; p<0.001			F=4.384; p=0.037	F=10.751; p=0.003
		10mg/kg										
	14d	0.4 mg/kg	F=31.342; p<0.001	F=26.277; p<0.001							F=15.215; p=0.002	
		10mg/kg										
	21d	0.4 mg/kg	F=25.475; p<0.001		F=6.921; p=0.013	*t*=2.957; *p*=0.018						
		10mg/kg										
	28d	0.4 mg/kg	F=12.934; p=0.002									
		10mg/kg										
Post-Exposure	7d	0.4 mg/kg							F=6.553; p=0.021		F=4.990; p=0.035	t=-3.752; p=0.007
		10mg/kg										
	14d	0.4 mg/kg				t=3.676; p=0.006		t=4.566; p=0.003	F=5.160; p=0.036			
		10mg/kg										

			Lipids		Carbohydr.		Proteins		Ea		Ec		CEA	
			20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC	20ºC	25ºC
Exposure	24h	0.4 mg/kg											F=4.991; p=0.031	F=10.725; p=0.003
		10mg/kg												
	48h	0.4 mg/kg			F=5.140; p=0.027							F=8.450; p=0.006		
		10mg/kg												
	96h	0.4 mg/kg			F=4.118; p=0.046	F=58.326; p<0.001		F=8.712; p=0.005		F=17.047; p<0.001		F=5.035; p=0.034	F=5.113; p=0.030	
		10mg/kg												
	7d	0.4 mg/kg						F=8.455; p=0.006				F=17.535; p<0.001		
		10mg/kg												
	14d	0.4 mg/kg												
		10mg/kg												
	21d	0.4 mg/kg	F=40.144; p<0.001	t=-2.813; p=0.023					F=14.276; p=0.003		F=5.271: p=0.031		F=13.959; p=0.002	t=-2.661; p=0.032
		10mg/kg												
	28d	0.4 mg/kg		t=3.262; p=0.014			F=4.256; p=0.046							
		10mg/kg												
Post-Exposure	7d	0.4 mg/kg					F=9.273; p=0.005							
		10mg/kg												
	14d	0.4 mg/kg												
		10mg/kg												

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Supplementary Data – Chapter IV

1. Materials and methods

1.1. Post-mitochondrial supernatant

The protocol to obtain the post-mitochondrial supernatant (PMS) that will be used for lipid peroxidation, glutathione *S*-transferases, glutathione peroxidase and catalase analysis was followed as described by Ferreira *et al.* (2010). Each replicate (two organisms) was homogenized using a sonicator (*Kika Labortechnik*, V200Scontrol, Germany) in 1ml K-Phosphate 0.1M buffer, pH 7.4. From the homogenate, 150µL were separated to a microtube and 5µL butylated hydroxytoluene (BHT) 4% in methanol were added for endogenous lipid peroxidation (LPO) determination. The remaining tissue homogenate (850 µL) was centrifuged at 10000g for 20 min. (4ºC) to isolate the Post-Mitochondrial Supernatant (PMS). The PMS was divided into four microtubes for posterior analysis of biomarkers and protein quantification. All microtubes were stored at -80ºC until analysis, for a period no longer than 2 weeks.

1.2. Lipid peroxidation

The lipid peroxidation (LPO) assay was based on the methods described by Bird and Draper (1984) and Ohkawa *et al.* (1979) and adapted to microplate by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 150 µL homogenated tissue, 500 µL trichloroacetic acid sodium salt (TCA) 12% (w/v), 500 µL 2-thiobarbituric acid (TBA) 0.73% (w/v) and 400µL Tris-HCl 60mM with diethylenetriaminepentaacetic acid **(**DTPA) 0.1mM. The reaction was carried out at 100ºC in a water bath for 1h. After this, samples were centrifuged for 5 min. at 11500 rpm (25ºC). Samples were kept away from light, at 25ºC and immediately read at 535 nm. LPO was

expressed as nmol TBARS hydrolyzed per minute per mg of wet weight, using a molar extinction coefficient of 1.56 x10 M^{-1} cm^{-1}.

1.3. Glutathione S-Transferases

Glutathione *S*-Transferases (GST) activity was determined based on the method described by Habig *et al.* (1974). The PMS (100µL) was added to 200µL of a reaction solution and the result/substrate produced was measured at 340 nm. The reaction solution was a mixture of 4.95 ml K-phosphate buffer 0.1M (pH 6.5) with 900µL L-glutathione reduced (GSH) 10mM, and 150µL 1-chloro-2,4-dinitrobenzene (CDNB) 10mM. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6 $x10^{-3}$ M^{-1} cm^{-1}.

1.4. Glutathione Peroxidase

Glutathione Peroxidase (GPx) activity was determined based on the method described by Mohandas *et al.* (1984). PMS (50µl) was mixed with 840µl K-phosphate buffer 0.05 M (pH 7.0), in a EDTA 1 mM solution, sodium azide 1mM and glutathione redutase (GR) (7.5mL from stock with 1 U/mL). Then 50 µL glutathione reduced (GSH) 4mM, NADPH and H_2O_2 (10 µl, 0.5mM) was added as substrate to the solution. The decrease in NADPH content (50µl, 0.8mM) was measured at 340 nm and the enzymatic activity expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 6.22x10^{-3} M^{-1} cm^{-1}.

1.5. Catalase

Catalase (CAT) activity was determined based on the method described by Clairborne (1985). PMS (50µl) was mixed with 500µL H_2O_2 0.030M, and 950µL K-Phosphate 0.05M (pH 7.0) and the decomposition of the substrate (H_2O_2) measured at 240 nm. The enzymatic

activity was expressed as unit (U) per mg of protein where a U corresponds to one µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

1.6. Acetylcholinesterase- sample preparation and reaction

One isopod head per sample was homogenized using a sonicator in 500µl of potassium phosphate buffer (0.1M, pH 7.2), and the supernatants obtained after centrifugation (4ºC, 3800g, 3 min) were removed and stored at -80ºC until enzymatic analysis. The AChE activity determination was performed according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996).

In a 96 well microplate 250 µl of the reaction solution was added to 50µl of the sample and the absorbance was read at 414 nm, after 10, 15 and 20min. The reaction solution had 1ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10mM solution, 1.280ml of 0.075M acetylthiocholine iodide solution and 28.920 ml of 0.1M phosphate buffer. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $1.36x10^{-3}$ M^{-1} cm^{-1}.

1.7. Protein quantification for biomarkers

For all biomarker, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

1.8. Energy reserves:

To determine total protein, carbohydrate and lipid contents, one isopod was homogenized using a sonicator in 1000µl distilled water after which was divided into three microtubes each one containing 300µl of the homogenate. The first fraction was used to determine

protein and carbohydrate content, the second fraction to determine lipid content and the third fraction to determine the electron transport activity (ETS).

To determine total protein and carbohydrate contents, the homogenate was mixed with 100µl of 15% trichloroacetic acid (TCA) and incubated at -20ºC for 10 min (adapted from de Coen and Janssen (1997). After centrifugation (1000g, 10 min, 4ºC), the supernatant was separated as well as the carbohydrate fraction. The remaining pellet was resuspended in 1250µl sodium hydroxide (NaOH), incubated at 60 ºC for 30 min, after which it was neutralised with 750µl hydrocloric acid (HCl) and used as the protein fraction. Total protein content was then determined using the Bradford's reagent (Bradford 1976), and by measuring the absorbance at 590 nm using bovine serum albumin as a standard. Total carbohydrate content was determined by adding 50µl of 5% phenol and 200µl sulphuric acid (H_2SO_4) to 50µl of sample in a multiwell microplate, incubated for 30min at 20 ºC; the absorbance was measured at 492 nm using glucose as a standard. The protein and carbohydrate content is expressed as mg/mg organism (org) and J/mg org (expressed as fresh weight).

Total lipid quantification was based in the method described by Bligh and Dyer (1959). To the homogenate obtained was added 500µl chloroform (spectrofotometric grade). After vortexed, 500µl methanol (spectrofotometric grade) and 250µl bi-distilled water were added, centrifuged (1000g, 5min, 4ºC) and the top phase removed; the remaining phase was used for lipid measurement. 100µl of lipid extract plus 500µl H_2SO_4 were heated for 15 min (200ºC); after cooling down, 1.5ml of bi-distilled water was added and the total lipid content determined by measuring the absorbance at 375 nm using tripalmitin as a standard. The lipid content was expressed as mg/ mg org and J/mg org (expressed as fresh weight).

The electron transport activity was measured following de Coen and Janssen (1997) with the following modifications. To the homogenate obtained was added 150 µL of a buffer (0.3 M Tris-HCl pH 8.5, 45% (w/v) Poly Vinyl Pyrrolidone, 459 µM $MgSO_4$ and 0.6% (w/v) Triton X-100). Samples were then centrifuged at 1300 g during 10 min (4ºC).

The electron transport activity was determined by adding 50 µL of sample to 150 µL buffered substrate solution (0.13M Tris HCl, 0.3% (w/v) Triton X-100, pH 8.5, 1.7 mM NADH and 250 µM NADPH). The reaction was started by adding 100 µL INT (p-IodoNitroTetrazolium; 8 mM) and the absorbance measured at 490nm for 3 min.

The amount of formazan formed was calculated using a molar extinction coefficient of $15900 M^{-1} cm^{-1}$.

1.9. Cellular Energy Allocation (CEA)

The different energy reserve fractions (Ea): protein, carbohydrate and lipids obtained for the individual organisms were transformed into energetic equivalents using the energy of combustion described by Gnaiger (1983): 17.5 J/mg carbohydrate, 24 J/mg protein and 39.5 J/mg lipid. The cellular respiration rate (Ec) was determined using the ETS data, based on the theoretical stoichiometric relationship that for each 2 µmol of formazan formed, 1 µmol of O2 was consumed in the ETS system. The oxygen consumed per isopod was transformed into energetic equivalents using the specific oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture of 484 kJ/mol O2 (Gnaiger, 1983). The Ea, Ec and CEA value were calculated as described by Verslycke *et al.* (2004):

Ea (available energy) = carbohydrates + lipids + proteins (mJ / mg org.)
Ec (energy consumption) = ETS activity (mJ / mg org. / h)
CEA (cellular energy allocation) = Ea/Ec

1.10. Integrated Biomarker Response (IBR)

To try to integrate all results from different biomarkers and understand global/general responses, the integrated biomarker response (IBR) was calculated according to Beliaeff and

Burgeot (2002). The IBR is calculated by summing up triangular Star Plot areas calculated for each two neighbouring data (biomarkers and energy reserves, time or temperature).

To calculate the IBR for biomarkers and energy reserves, the general mean (m) and the standard deviation (s) of all data regarding a given biomarker was calculated, followed by a standardization for each situation to obtain Y, where $Y = (X - m)/s$, and X is the mean value for the biomarker at a given concentration. Then Z was calculated using $Z = -Y$ or $Z = Y$, in the case of a biological effect corresponds respectively to an inhibition or a stimulation. Regarding the biological effect considered for each parameter, AChE and the energy related parameters: lipids, carbohydrates, proteins, energy available (Ea) and CEA were all assumed to decrease upon nickel exposure. In a similar way LPO rate was always assumed to increase with the exposure to the metal. The energy consumption (Ec) and LDH can either increase or decrease depending on the intensity of the stressor, and with organisms' strategy as well. In theory, organisms tend to spend more energy in order to deal with stressor, but an opposite strategy can also be used. In this case, organisms tend to decrease the energy that is directed to other physiological processes and thus resulting in an overall lower consumption, even lower than in situations where they were not under stress. In a similar way, the activity of the biomarkers GST and CAT can also be induced in order to cope with the formation of lipid peroxides or inactivated by ROS-mediated denaturation (Lizawa et al. 1994). For these reasons, their kinetics must be followed through time to consider their biological effect.

The score (S) was calculated by $S = Z+|Min|$, where $S \geq 0$ and $|Min|$ is the absolute value for the minimum value for all calculated Y in a given biomarker at all measurements made.

Star plots were then used to display Score results (S) and to calculate the integrated biomarker response (IBR) as:

$$IBR = \sum_{i=1}^{n} A_i$$

$$A_i = \frac{S_i}{2} \sin\beta \, (S_i \cos\beta + S_{i+1} \sin\beta)$$

$$\beta = \tan^{-1}\left(\frac{S_{i+1}\sin\alpha}{S_i - S_{i+1}\cos\alpha}\right)$$

where S_i and S_{i+1} are two consecutive clockwise scores (radius coordinates) of a given star plot; A_i corresponds to the area the connecting two scores; n the number of biomarkers and energy reserves used for calculations; and $\alpha = 2\pi/n$.

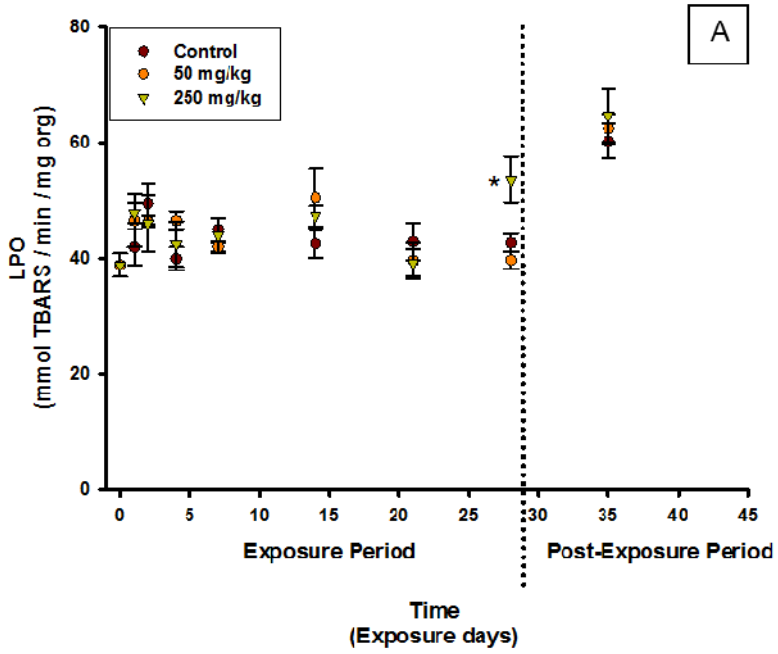
In some sampling times, due to the high mortality obtained, it was not possible to determine all the parameters. Since the IBR is obtained by summing up all the parameters, to allow a correct and more accurate comparison it was divided by the number of sampling times and presented as IBR/n (Broeg and Lehtonen, 2006). Using this method it is possible to get an overall state of organisms for each parameter and each sampling time.

Analysing the scores as a fitness index, values that differed in 0.5 from the control score were considered to be from an animal with a higher or lower fitness (higher or lower scores, respectively).

The IBR calculations were always performed with the same order of parameters for all sampling times: the neurotoxicity biomarker AChE, followed by the detoxification and oxidative stress biomarkers GST, LPO and CAT, then the energy related biomarker LDH to serve as transition between biomarkers and energy related parameters and finally the lipids, carbohydrates and proteins content, the energy available (Ea), the energy consumption (Ec) and the CEA that integrates the last two parameters.

2. Results

2.1. Figures



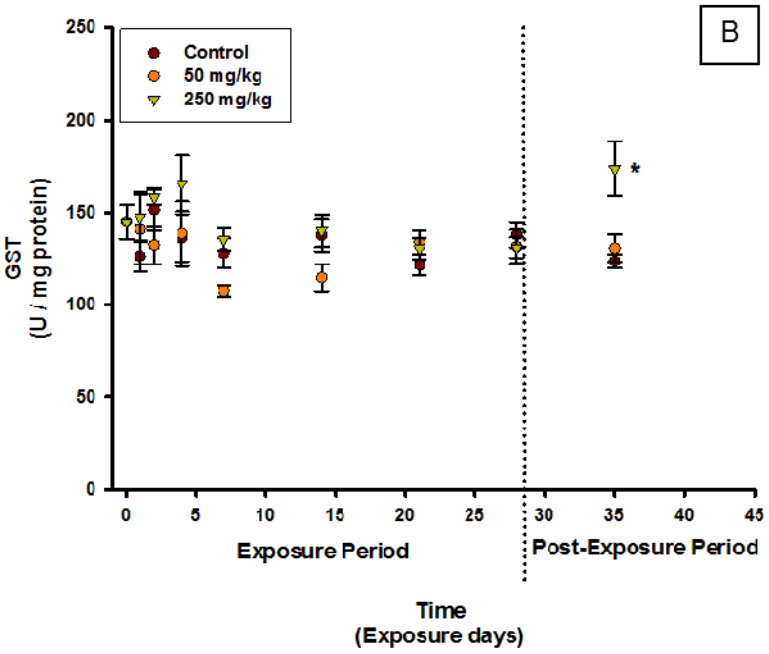
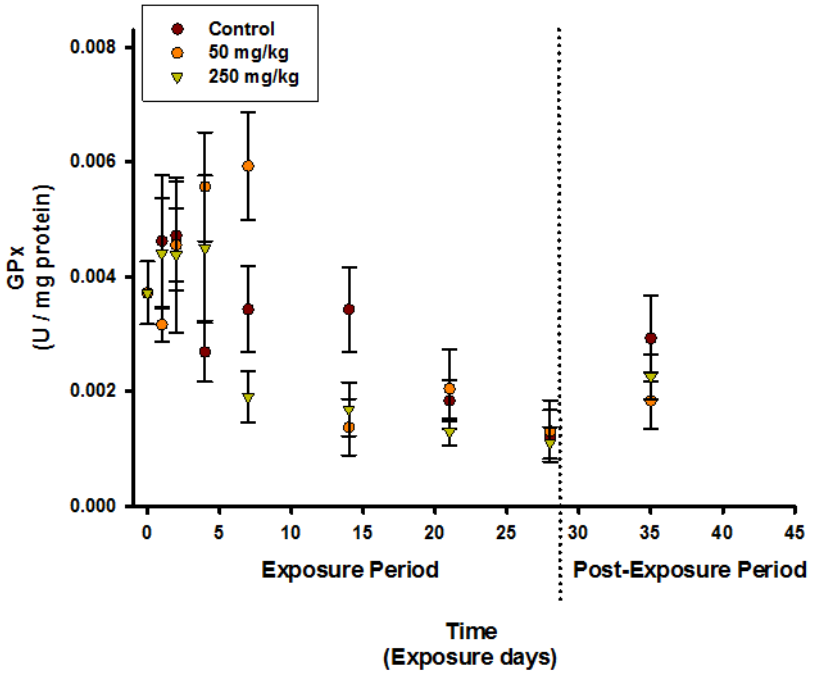
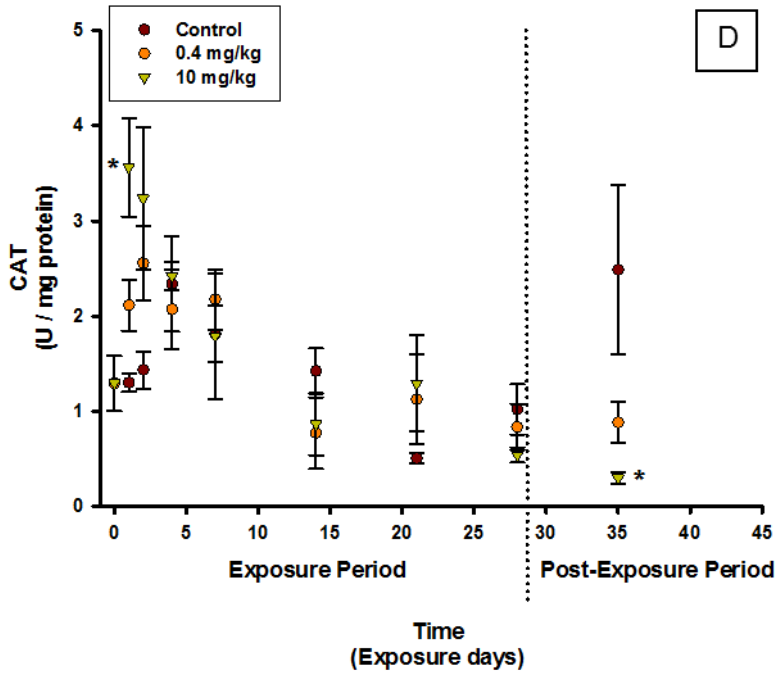


Fig. 1SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed to nickel (Δ:50 mg / kg soil and □:250 mg / kg soil) for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$





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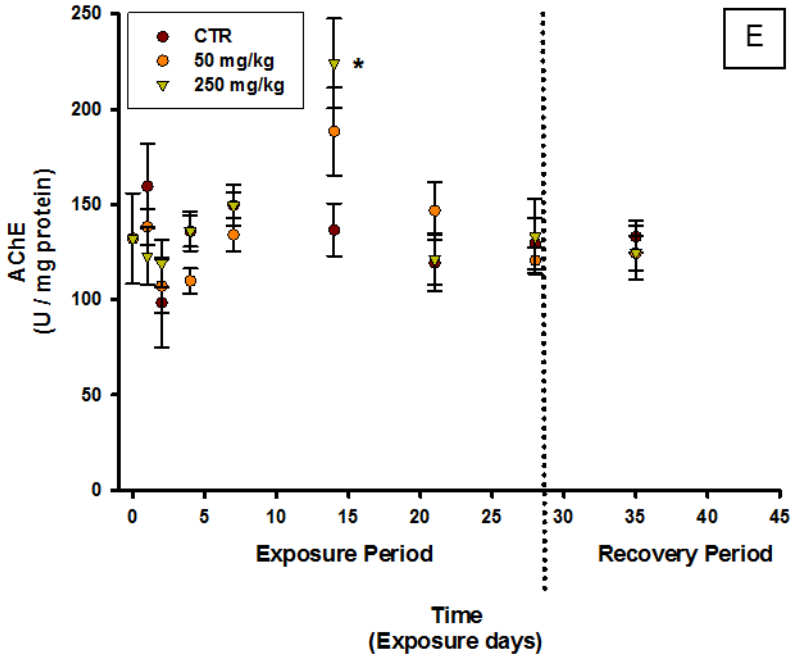

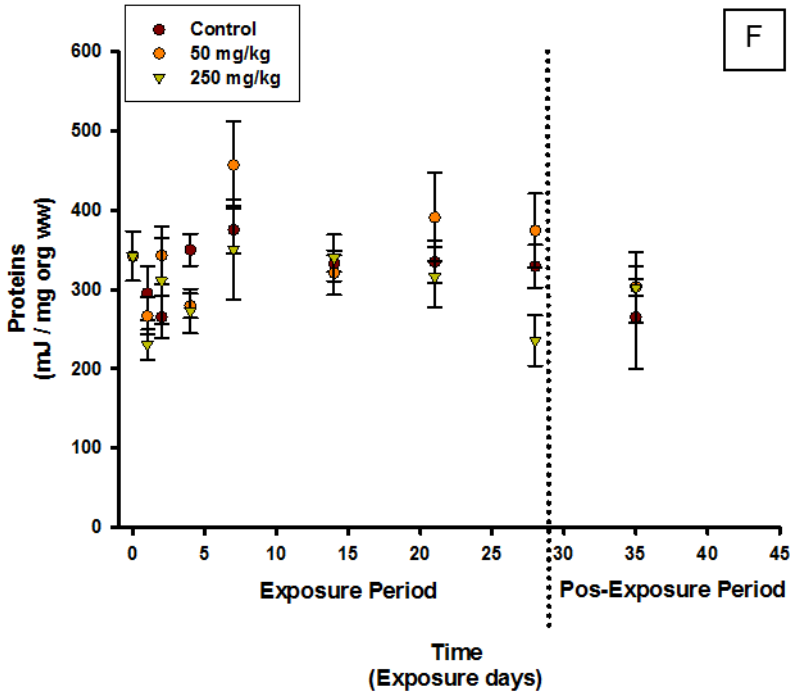

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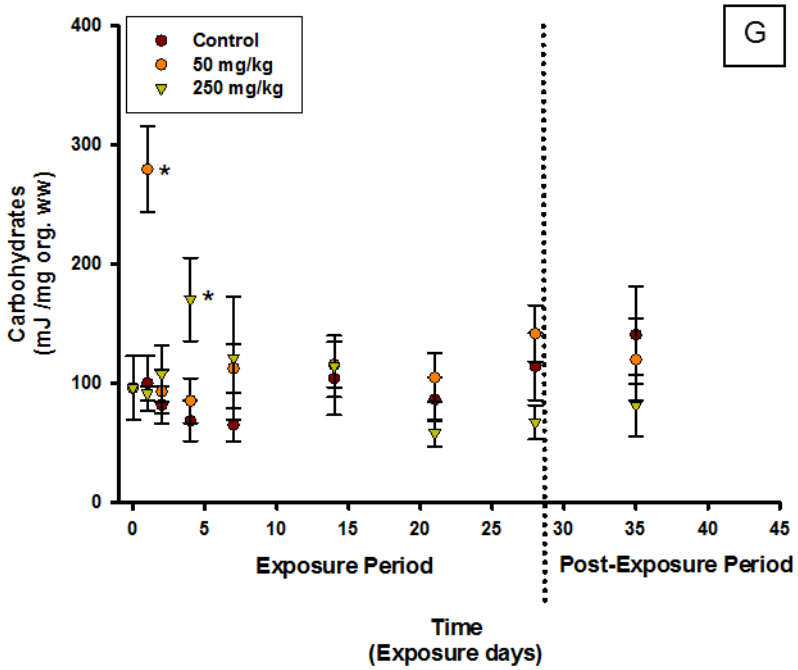

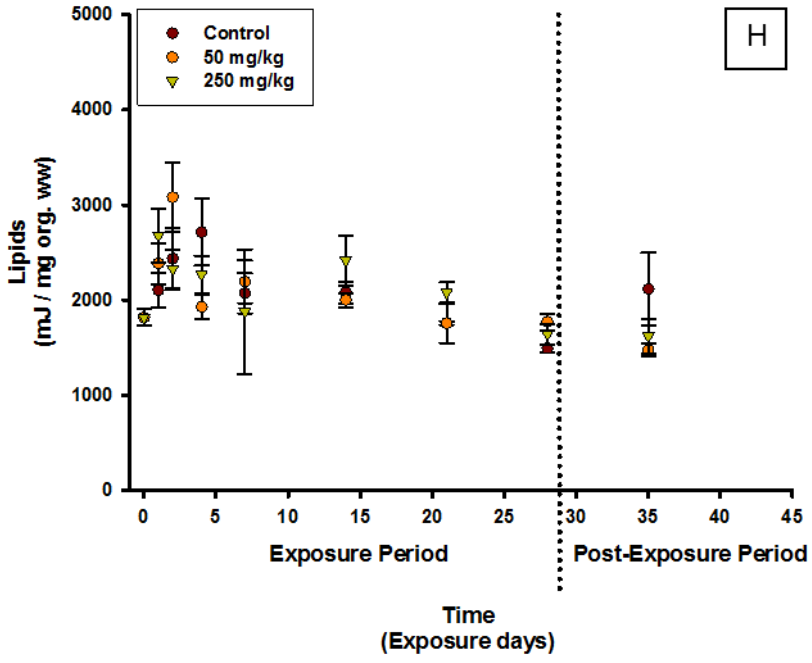

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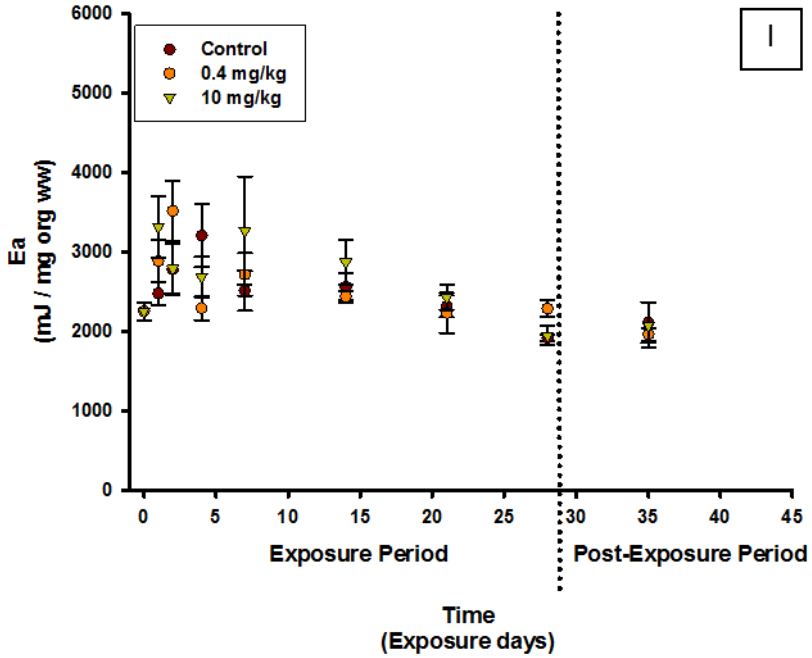

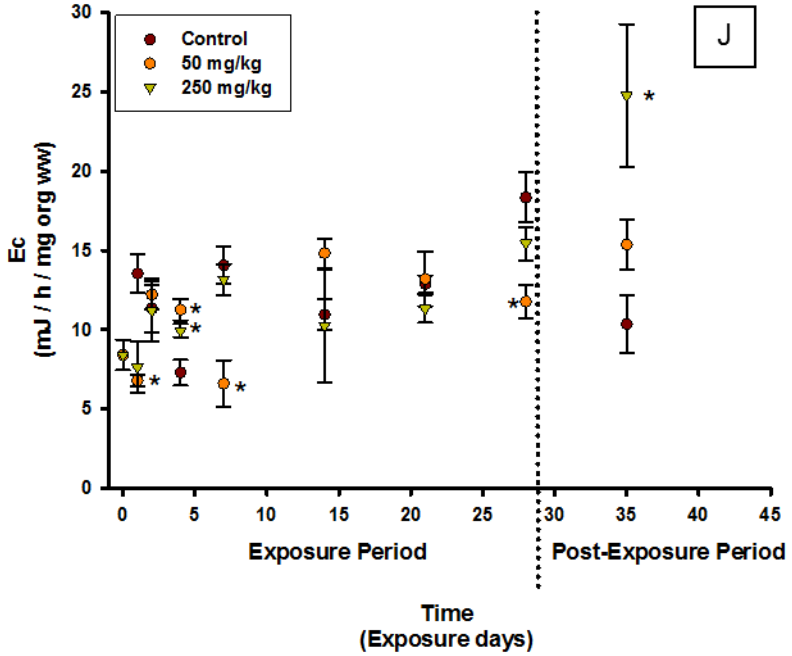

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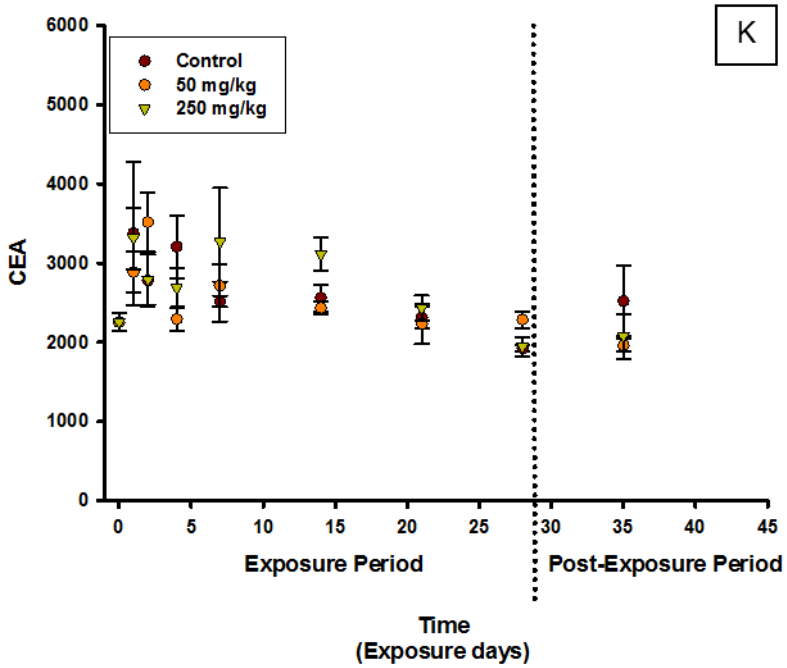

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2.2. Tables

Table 1SD IBR data comparison for biomarkers and energy reserves between control organisms from the species Porcellionides *pruinosus* and those exposed to nickel. Data refers to a 28 day exposure period followed by a 14 day recovery period. Red boxes denote deleterious effects, green boxes denote positive effects. □ - denotes increase, □ - denotes decrease; IBR is ± 0.5 from control's IBR value.

			AChE	GST	LPO	CAT	GPx	Lipids	Carbohydrates	Proteins	Ea	Ec	CEA
Exposure	24h	50 mg/kg			↘				↗			↗	↘
		250 mg/kg	↘	↘	↘	↗		↗		↘	↗	↗	
	48h	50 mg/kg		↗		↗		↗		↗	↗		↗
		250 mg/kg		↗		↗							
	96h	50 mg/kg	↘		↘		↘	↘		↘	↘	↘	↘
		250 mg/kg		↘			↘	↘	↗	↘			↘
	7d	50 mg/kg		↗			↘		↗	↗		↗	
		250 mg/kg					↗		↗		↗		↗
	14d	50 mg/kg	↗	↗	↘		↘						
		250 mg/kg	↗		↘		↘						↗
	21d	50 mg/kg								↗			
		250 mg/kg											
	28d	50 mg/kg										↗	
		250 mg/kg			↘				↘	↘			
Post-Exposure	35d	50 mg/kg				↗		↘				↘	↘
		250 mg/kg		↘	↘	↗		↘	↘			↘	

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Supplementary Data – Chapter VI

1. Results

1.1. RNA-Seq analysis: Ni 50 mg/kg soil

The KEGG pathway analysis revealed that a total of 22 pathways were being impacted. The upregulated genes impacted 15 pathways with the most impacted being: Amino sugar and nucleotide sugar metabolism (pathway ID 00520); Other glycan degradation (pathway ID 00511) and Glycerolipid metabolism (pathway ID 00561). The dowregulated genes impacted 10 pathways, all at the same. In Fig. 1SD is showed a general diagram of metabolic pathways being affected by 50 mg Ni/kg soil extracted from KEGG database.

In Table 1SD are presented the top five annotated transcripts that suffer the most alterations for each concentration.

Table 1SD Top 5 downregulated and upregulated annotated transcripts for exposure to Ni 50mg/kg soil.

Fold change	Uniprot ID	Gene homology
Downregulation		
-6.0667	P03646	Minor spike protein H
-6.0054	Q02357	Ankyrin-1
-5.8668	P69487	Scaffolding protein D
-5.7069	P07928	A' protein
-5.4525	P07932	Major spike protein G
Upregulation		
7.5577	O43451	Maltase-glucoamylase, intestinal
7.2203	Q9U572	Hemolymph cottable protein
6.6278	P91778	Alpha-amylase
6.5900	Q02157	Pancreatic triacylglycerol lipase
6.3992	O6P7A9	Lysosomal alpha-glucosidase

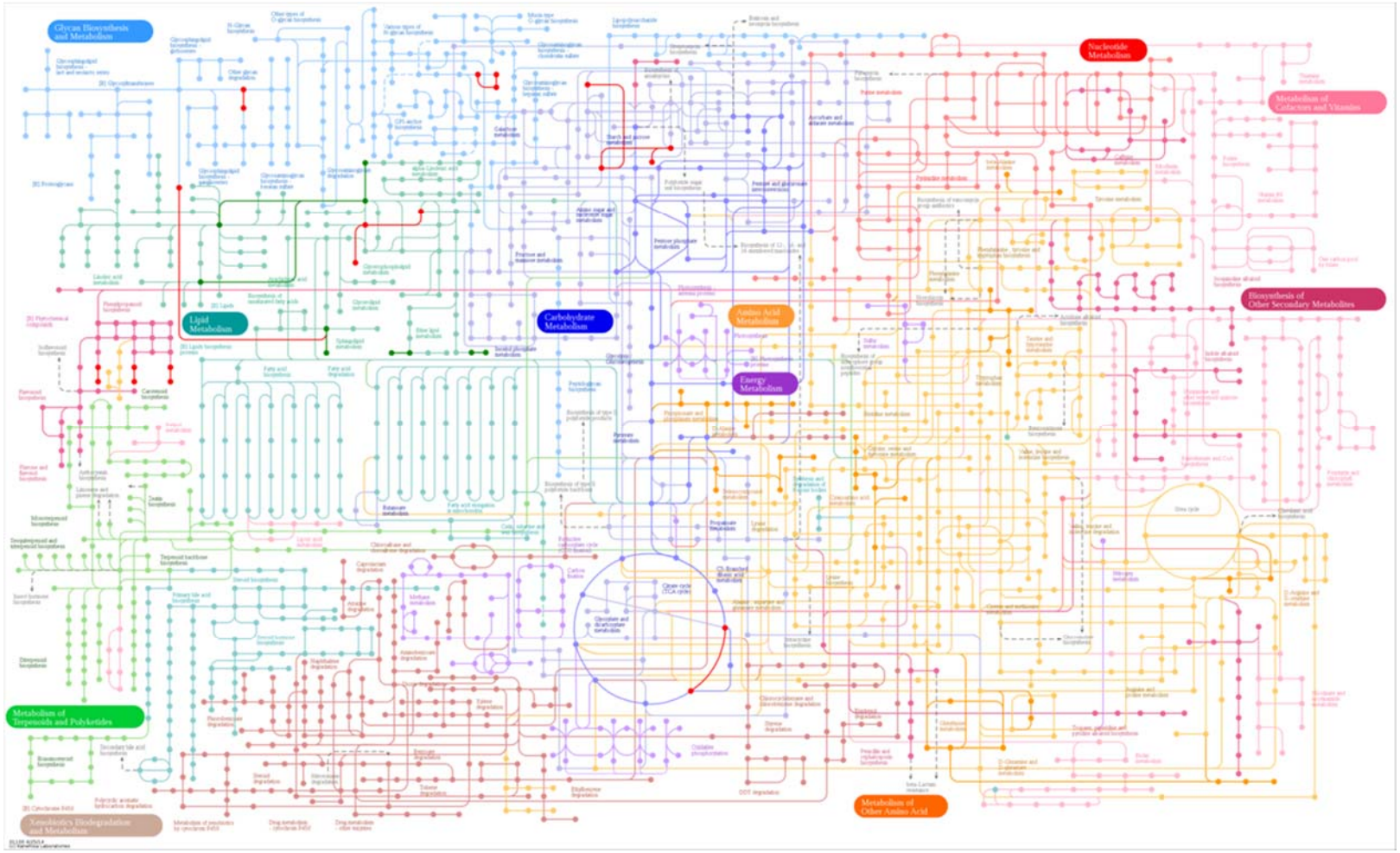

Fig. 1SD General diagram of metabolic pathways being affected by 50 mg Ni/kg soil extracted from KEGG database. Green dots and lines denote significant upregulated genes; Red dots and lines denote significant downregulated gene

The transcripts for the exposure to the lower concentration of nickel (50 mg/kg soil – Fig. 3SD) show a similar distribution between up and downregulated processes. For biological process (approx. 45%) localization and growth do not appear in downregulated transcripts. As for the molecular function (approx. 30%), upregulated trasncripts do not inclued receptor activity whereas downregulated transcritpts do not inclued transporter activity, nucleic acid binding transcription factor activity, antioxidant activity, molecular transducer activity, electron carrier activity or enzyme regulator activity. In the cellular components (15 to 20%) the downregulated transcripts do not include extracellular region or membrane-enclosed lumen.

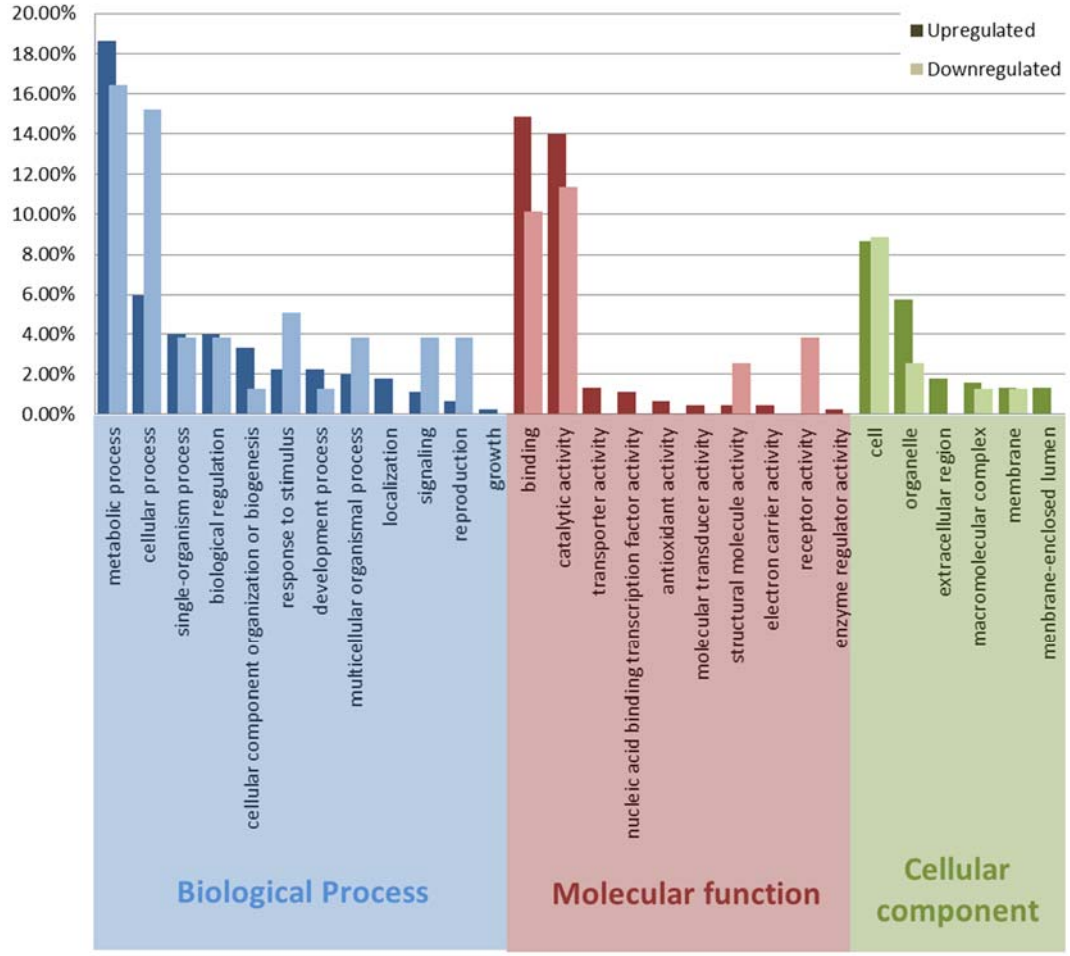

Fig. 2SD Distribution of GO classifications of significant up and downregulated transcripts into three main categories: biological process, cellular component and molecular function and their subcategories. Column heights are the percentage of annotated transcripts that mapped to each correspondent GO term. Darker bars represent upregulated transcripts and lighter bars represent downregulated transcripts.

1.2. RNA-Seq analysis: Ni 250 mg/kg soil

The KEGG pathway analysis revealed that a total of 13 pathways were being impacted. The upregulated genes impacted 7 pathways with the most impacted being the Amino sugar and nucleotide sugar metabolism (pathway ID 00520. The dowregulated genes impacted 6 pathways, all at the same. In Fig. 4SD is showed a general diagram of metabolic pathways being affected by 250 mg Ni/kg soil extracted from KEGG database.

In Table 2SD are presented the top five annotated transcripts that suffer the most alterations for each concentration.

Table 2SD Top 5 downregulated and upregulated annotated transcripts for exposure to Ni 250mg/kg soil.

Fold change	Uniprot ID	Gene homology
Upregulation		
-6.4815	P07932	Major spike protein G
-6.0973	P03641	Capsid protein F
-5.8099	P07928	A' protein
-5.6372	Q6NUT3	Major facilitator superfamily domain containing protein 12
-5.6175	P03646	Minor spike protein H
Downregulation		
8.7822	Q86T26	Transmembrane protease serine 11B
8.1253	P20214	Neuroglian
6.6222	Q0IIH7	Suppressor tumorigenicity 14 protein homolog
6.5738	Q5RAG8	Prolyl 4-hydroxylase subunit alpha-1
6.3503	Q9W5U2	Chitinase 3

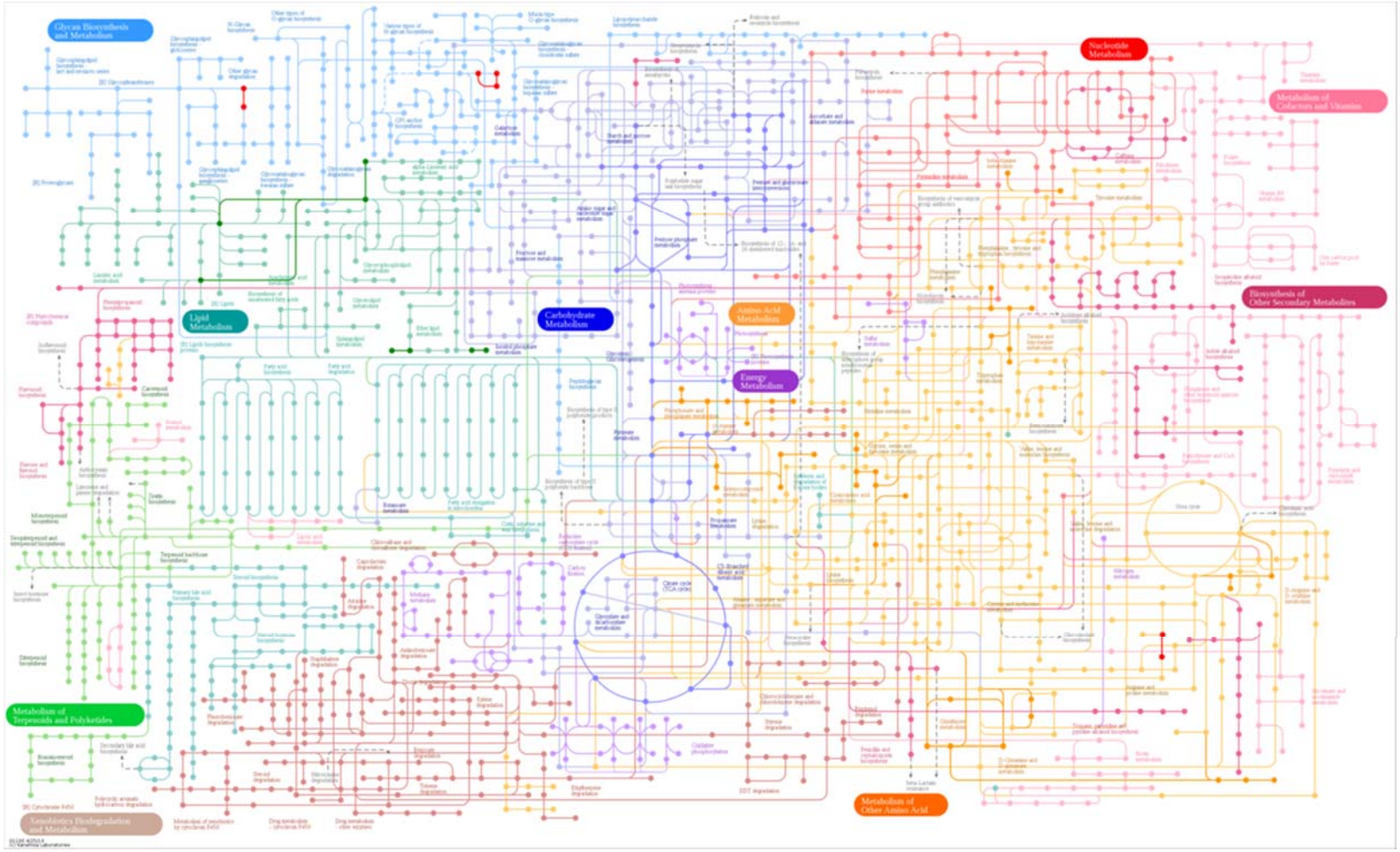

Fig. 3SD General diagram of metabolic pathways being affected by 250 mg Ni/kg soil extracted from KEGG database. Green dots and lines denote significant upregulated genes; Red dots and lines denote significant downregulated gene

The distribution analysis of GO categories for the significant up and downregulated transcripts to control, in the higher concentration of Ni (250 mg/kg soil) is presented in Fig. 4SD. A percentage of 43.11% of the upregulated transcripts were found to be involved in biological processes, 35.46% in molecular functions and 21.43% in cellular components, whereas the downregulated transcript were in its majority involved in biological processes (62.32%), approx. 30% in molecular function and only 10%in cellular components. Within the biological processes no upregulated transcripts were related to reproduction, whereas in molecular function no downregulated transcripts were found to be involved in molecular transducer activity or enzyme regulator activity. Also within the cellular components downregulated transcripts were only present in cell and organelles.

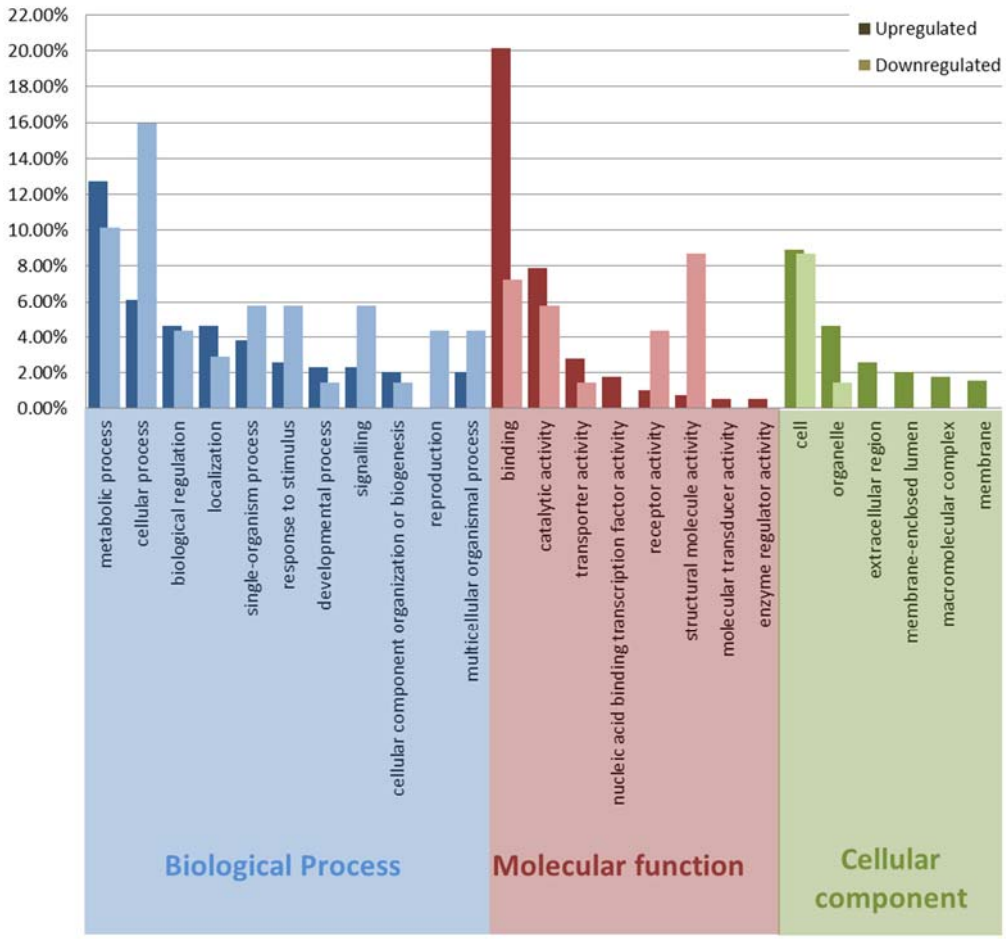

Fig. 4SD Distribution of GO classifications of significant up and downregulated transcripts for organisms exposed to 250 mg/kg soil into three main categories: biological process, cellular component and molecular function and their subcategories. Column heights are the percentage of annotated transcripts that mapped to each correspondent GO term. Darker bars represent upregulated transcripts and lighter bars represent downregulated transcripts.

1.3. RNA-Seq analysis: Nickel treatments' comparison

In Fig. 5SD is presented the RNA-Seq analysis for the exposures to nickel, and the up/down regulated annotated transcripts common to both concentrations (50 and 250 mg/kg soil) and respective GO classifications.

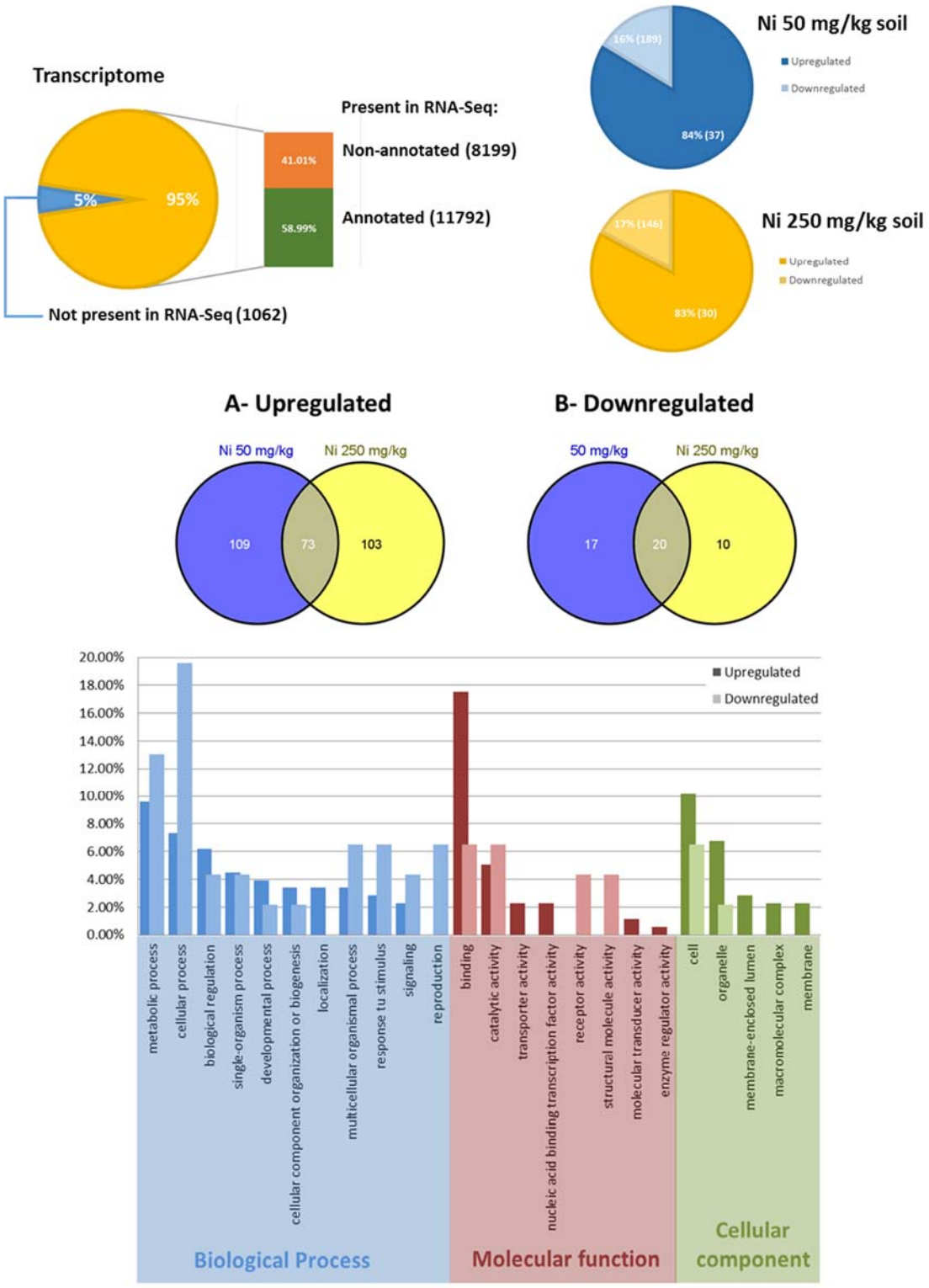

Fig. 5SD Transcriptome and the number of transcripts that were counted in RNA-Seq analysis, along with the percentage of annotated and non-annotated number. Nickel exposures to 50 and 250 mg/kg soil and respective up and downregulated transcripts along with Venn diagrams' showing the differentially expressed transcripts ($p < 0.05$) that are unique and common between treatments (A- upregulated transcripts; B- downregulated transcripts). Diagram with the distribution of GO classifications of significant up and downregulated transcripts into three main categories: biological process, cellular component and molecular function and their

subcategories. Upregulated transcripts are always assigned into dark colors and downregulated transcripts are always assigned into lighter colors. Transcript numbers always represent annotated transcripts, except for the first chart.

The analysis between Ni 50mg/kg and Ni 250mg/kg showed three transcripts significantly expressed: RING finger protein (-4.87 and -4.70 fold to control, respectively for Ni 50 mg/kg soil and Ni 250 mg/kg soil); Major facilitator superfamily domain-containing protein 12 (-3.89 and -4.29 fold to control, respectively for Ni 50 mg/kg soil and Ni 250 mg/kg soil) and Macrophage mannose receptor 1 (4.02 and 6.23 fold to control, respectively for Ni 50 mg/kg soil and Ni 250 mg/kg soil).

The common transcripts for the exposure to Ni show that upregulated approximately half of the transcript are involved in biological process (46.89%) and the other half is divided into molecular function (28.81%) and cellular components (24.29%), whereas the downregulated transcripts are mainly involved in biological process (69.57%), followed by molecular function (21.74%) and cellular component (8.70%). Within the biological processes, reproduction (3; 6.52%) only appears related to downregulated transcripts, whereas localization (6; 3.39%) is only related to upregulated transcripts. In molecular functions, receptor activity (2; 4.35%) and structural molecule activity (2; 4.35%) are only related to downregulated transcript, whereas transporter activity (4; 2.26%), nucleic acid binding transcription factor activity (4; 2.26%), molecular transducer activity (2; 1.13%) and enzyme regulator activity (1; 0.56%) are only related to upregulated transcripts. In the cellular components downregulated transcripts are only related to cell (3; 6.52%) and organelle (1; 2.17%) whereas upregulated transcripts are present also in membrane-enclosed lumen, macromolecular complex and membrane.